ANTIANGIOGENIC STRATEGIES FOR THE TREATMENT OF HAEMATOLOGICAL MALIGNANCIES

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Declaration

I, Reuben Benjamin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Angiogenesis plays a key role in the pathogenesis of haematological malignancies such as acute myeloid leukaemia (AML), multiple myeloma (MM) and lymphoma (NHL). The evidence supporting this theory includes the finding of increased bone marrow microvessel density and increased levels of plasma and urinary pro-angiogenic cytokines in patients with these malignancies as well as encouraging results from preclinical and clinical studies using antiangiogenic therapies. A significant limiting factor in most of these studies has been the short half-life of most antiangiogenic compounds which has necessitated the use of cumbersome administration protocols resulting in poor compliance and an inability to administer the drug long enough to result in its desired therapeutic effect. The aim of this thesis therefore is to investigate the therapeutic effects of stable antiangiogenic expression in xenograft models of AML, NHL and MM. We investigated the antitumour effects of stably blocking the vascular endothelial growth factor (VEGF) pathway, the matrix metalloproteinase pathway and the use of interferons, a family of cytokines with significant antitumour activity resulting not only from their antiangiogenic properties but also from their anti-proliferative, anti-apoptotic and immunomodulatory effects. Murine models of AML, NHL and MM were developed by engrafting a number of well characterized tumour cell lines – HL-60, Raji and KMS12-BM respectively into $\beta 2m^{null}$ NOD/SCID immunodeficient mice, resulting in a tumour phenotype resembling the human disease. Blockade of the VEGF pathway was achieved by stably expressing the soluble form of the VEGF receptor (sFlk-1) using adeno associated viral vectors or by using an antibody directed against VEGF but neither strategy resulted in antitumour activity in AML or NHL xenograft models. We were however able to show that stable expression of tissue inhibitor of metalloproteinase-3 (TIMP-3) resulted in significant antitumor activity against MM cells in vivo. Finally we were able to demonstrate for the first time that Interferon-β has profound antileukaemic activity in the setting of a xenograft model and that even low level expression when stable can significantly inhibit primary AML engraftment in vivo.

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INDEX

FIGURE INDEX

Chapter 1

Chapter 3

Chapter 4

TABLE INDEX

LIST OF ABBREVIATIONS

AAV	Adeno associated virus
AML	Acute myeloid leukaemia
BM	Bone marrow
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorting
GFP	Green Fluorescent protein
hFIX	Human factor IX
IFN	Interferon
IU	Infectious units
MMPs	Matrix metalloproteinases
MM	Multiple myeloma
MOI	Multiplicity of infection
MVD	Microvessel density
mFlk-1	Murine fetal liver kinase (VEGF receptor-2)
μl	Microlitre
μg	Microgram
NOD	Non obese diabetic
PCR	Polymerase chain reaction
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
TIMPs	Tissue inhibitor of matrix metalloproteinases
TU	Transducible units
VG	Vector genomes
VEGF	Vascular endothelial growth factor

CHAPTER 1 : INTRODUCTION

1.1 Angiogenesis - role in tumourigenesis

Angiogenesis, the process of blood vessel formation from pre-existing vessels is essential for the female reproductive cycle, for tissue repair and wound healing. In addition many diseases are driven by persistent unregulated angiogenesis namely diabetic retinopathy, arthritis and ocular neovascularisation. It is now also clear that the growth and metastatic potential of most tumours, both solid and haematological, are angiogenesis dependent.

Most tumours can persist in situ for a considerable period of time ranging from months to years without neovascularisation. Folkman first described the phenomenon by which dormant tumours become vascularised when a subgroup of cells within the tumour acquire an angiogenic phenotype. According to Folkman tumours upto 2-3 mm^3 can exist in a prevascular phase but in order to further increase in size they require neovascularisation (Folkman, 1990). The switch to the angiogenic phenotype occurs as a result of a change in the local equilibrium between pro and antiangiogenic factors in the vicinity of the tumour. During this process proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) are overexpressed by tumour or stromal cells. In addition many of the endogenous inhibitors of angiogenesis such as angiostatin, interferons and tissue inhibitors of metalloproteinases (TIMPs) are downregulated. The net effect is to promote endothelial cell migration and proliferation. The tumour vasculature delivers oxygen and nutrients to proliferating tumour cells as well as releasing growth factors and cytokines, both of which facilitate tumour growth and metastases.

1.2 Angiogenesis – role in haematopoiesis and haematological malignancies

It is now recognised that the haematopoietic and vascular systems are linked closely by virtue of the fact that both systems appear to be derived from a common precursor called the haemangioblast which originates from the mesoderm in the developing embryo (Choi et al., 1998). Evidence supporting this hypothesis comes from studies showing that haematopoietic and endothelial cells share expression of a number of genes such as CD34, Flk-1, Flt-1, Tie-2 and SCL/TAL-1. Overexpression of the SCL/TAL-1 gene leads to excess production of both haematopoietic and endothelial cells in zebrafish models (Gering et al., 1998). CD34 enriched human peripheral blood cells were shown to have the ability to differentiate into endothelial cells in vitro (Asahara et al., 1997). In addition Shi et al were able to demonstrate that BM derived human CD34+ peripheral blood cells had the potential to differentiate into endothelial cells in a canine bone marrow

transplantation model (Shi et al., 1998). Asahara et al went one step further and showed that BM derived endothelial cell (EC) progenitors contributed to postnatal physiologic as well as pathological neovascularisation (Asahara et al., 1999). In these experiments syngeneic transgenic mice expressing β-galactosidase under the transcriptional regulation of an endothelial specific promoter were used as transplant donors. EC progenitors were identified in the endometrium of transplanted mice following induction of ovulation as well as in the vicinity of cutaneous wounds and in association with subcutaneously implanted colon carcinoma tumour cells.

The process of angiogenesis in haematological malignancies is similar to that seen in solid tumours. Endothelial cells from pre-existing venules within the bone marrow are activated, proliferate, migrate and form new blood vessels which in turn supports tumour proliferation. Complex interactions take place between the neovasculature, tumour cells and the extracellular matrix mediated by growth factors, cytokines and by cell-cell contact in the bone marrow. Detailed evidence supporting the role of angiogenesis in acute myeloid leukaemia, myeloma and lymphoma includes the finding in these conditions of increased microvessel density and elevated levels of pro-angiogenic cytokines and the antitumour effects of antiangiogenic therapies, all of which are discussed in detail later in this chapter.

1.3 Factors involved in angiogenesis

A large number of pro and anti-angiogenic factors have been identified which play a role in regulating physiological and tumour induced angiogenesis (reviewed in (Bouis et al., 2006)). Two of the more important groups of angiogenic factors - VEGF and MMPs will be discussed in more detail in this chapter.

1.3.1 Vascular endothelial growth factor

Vascular endothelial growth factor is the most well characterized proangiogenic factor and has atleast six isoforms (VEGF, PIGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E), all of which are secreted as dimeric glyoproteins. They act through specific VEGF receptors - VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 as well as the accessory receptors Neuropilin-1 and Neuropilin-2.

VEGF is one of the most potent proangiogenic factors known and has high specificity for the vascular endothelium. It is essential for the early development of the vasculature during embryogenesis with homozygous VEGF knockout mice showing embryonic

lethality due to defects in blood island formation (Ferrara, 2004). Furthermore knockout of the VEGFR-2 gene also leads to absence of blood vessels and non survival of the embryo (Shalaby et al., 1995) while absence of the VEGFR-1 gene results in severe impairment of functional blood vessel development (Fong et al., 1995). VEGF plays a major role in stimulating physiological angiogenesis. For instance development of retinal vasculature has been shown to be mediated by hypoxia induced VEGF expression by surrounding neuroglial cells (Stone et al., 1995). In addition VEGF induced angiogenesis plays an important role in wound healing (Brown et al., 1992) and in the pathogenesis of disorders such as diabetic retinopathy (Adamis et al., 1994). VEGF also appears to be the main inducer of tumour angiogenesis. Tumour hypoxia and oncogenes upregulate VEGF levels in the neoplastic cells as well as upregulate VEGFR-1 and VEGFR-2 receptors on tumour endothelial cells the net effect of which is to promote tumour progression. VEGF contributes to tumour angiogenesis firstly by stimulating endothelial cell proliferation and migration and secondly by rendering vessels hyperpermeable to plasma proteins leading to their extravastion and the formation of a matrix that supports blood vessel growth (Dvorak et al., 1999). There is now extensive evidence implicating VEGF in tumour angiogenesis related to myeloma, acute myeloid/lymphoid leukaemia and lymphoma which will be summarized later on in this chapter. Some of the factors that potentiate VEGF production include hypoxia-inducible factor (HIF-1), fibroblast growth factor, platelet derived growth factor, transforming growth factor-β, keratinocyte growth factor and interleukin-6. On the other hand cytokines such as IL-10 and IL-13 inhibit the release of VEGF.

The biological activity of VEGF is mediated primarily through the VEGFR-1 and VEGFR-2 receptors. Both these receptors along with VEGFR-3 are transmembrane receptors with extracellular immunoglobulin like domains that bind VEGF and an intracellular tyrosine kinase domain through which the signaling cascade is activated. VEGFR-1 and VEGFR-2 are expressed predominantly on endothelial cells in contrast with VEGFR-3 which is found on lymphatic vessels. VEGFR-1 is also expressed on trophoblast cells, monocytes and renal mesangial cells whilst VEGFR-2 is also found on haematopoietic stem cells, megakaryocytes, retinal progenitor cells. Activation of both VEGFR-1 and -2 receptors results in endothelial cell migration although only VEGFR-2 activation appears to induce endothelial cell proliferation. Neuropilin-1 is also important for angiogenesis by acting as a VEGF coreceptor which potentiates the binding of VEGF to VEGFR-2 resulting in an enhanced endothelial cell migratory response (Soker et al.,

1998). Similarly neuropilin-2 has been shown to be a coreceptor for certain VEGF isoforms, again enhancing their biological response (Favier et al., 2006).

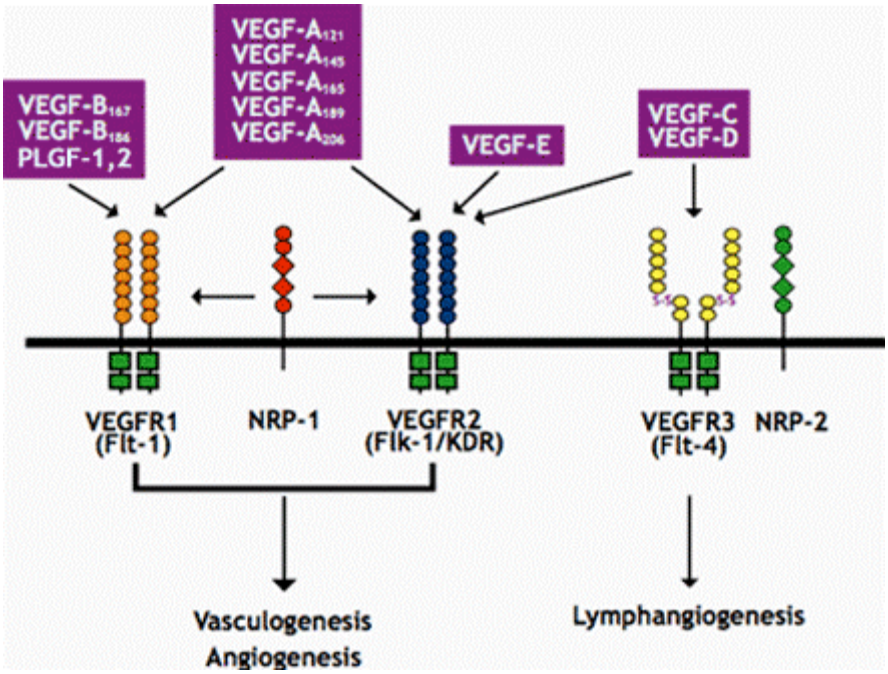
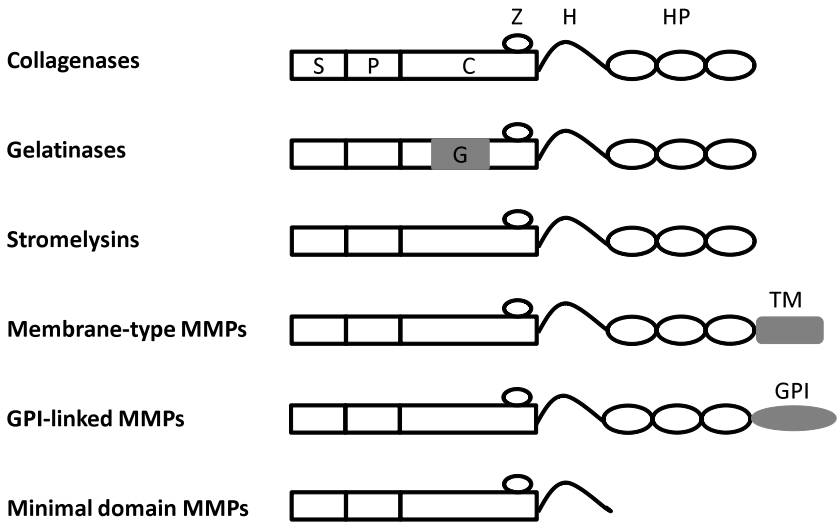


Figure 1.1 Schematic diagram showing interaction of VEGF ligands with their receptors - taken from (Hicklin and Ellis, 2005)

1.3.2 Matrix metalloproteinases and Tissue inhibitors of metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc dependent proteolytic enzymes that degrade components of the extracellular matrix thereby facilitating endothelial cell migration and tubule formation. There are over 25 MMPs all of which share a common catalytic domain but with varying specificities determined by a regulatory subunit, the hemopexin domain. Five major groups can be seen based on their substrate specificity – collagenases, gelatinases, stromelysins, membrane-type metalloproteinases and the others. The majority of the MMPs are secreted as inactive proteins which are activated following cleavage of the N-terminal pro-domain. Tissue inhibitors of matrix metalloproteinases (TIMPs) regulate the activity of the MMPs. Four TIMPs (TIMP 1-4) have been identified each of which has a different pattern of MMP inhibition.



S = signal peptide, P = prodomain, C = catalytic domain, Z = zinc site,
H = hinge region, HP = haemopexin domain, G = gelatin binding site,
TM = transmembrane domain, GPI = glycosylphosphatidyl inositol domain

Figure 1.2 Schematic diagram showing the domain structure of the matrix metalloproteinases

The primary function of MMPs is to break down the extracellular matrix barrier which enables endothelial cells as well as tumour cells to invade through the stroma (Nagase and Woessner, 1999). MMPs can also stimulate endothelial cell proliferation either directly

(Nishizuka et al., 2001) or by releasing growth factors like VEGF sequestered in the extracellular matrix (Bergers et al., 2000). They also affect endothelial cell attachment by cleaving vascular endothelial cadherin which plays a crucial role in cell-cell adhesion (Herren et al., 1998). MMPs can paradoxically also have an antiangiogenic effect by releasing angiogenesis inhibitors such as angiostatin from the extracellular matrix (Cornelius et al., 1998).

The gelatinase family of MMPs which include MMP-2 and MMP-9 have been studied extensively with considerable evidence supporting their integral involvement in tumour related angiogenesis. A number of studies have linked elevated levels of MMP-2 and MMP-9 to increased metastasis and tumour stage (Hanemaaijer et al., 2000; Schmalfeldt et al., 2001). MMP-2 and 9 expression is increased in invasive cell lines compared with non invasive types. Overexpression of MMP-2 and MMP-9 increased tumour metastasis in immunodeficient mice. In addition suppression of MMPs using recombinant TIMPs or synthetic MMP inhibitors resulted in inhibition of tumour growth and metastasis.

TIMPs are the endogenous regulators of MMPs and the balance between the level of active MMPs and TIMPs helps determine the extent of angiogenesis. All four known TIMPs inhibit specific MMPs with TIMP-3 having a broader spectrum of action that includes the ADAM family of metalloproteinases. TIMPs also have the ability to both promote as well as inhibit apoptosis of a number of cell types including tumour cells. TIMP-1 for instance enhances the proliferation of keratinocytes, fibroblasts and Raji lymphoma cells (Hayakawa et al., 1992). Timp-2 also has similar growth promoting properties (Hayakawa et al., 1994). On the other hand overexpression of TIMP-1 or -2 results in a reduction in tumour growth presumably because their MMP inhibitory properties come into effect (Gomez et al., 1997). Overexpression of TIMP-3 induces apoptosis of a number of tumour cell lines and vascular smooth muscle cells (Ahonen et al., 1998; Baker et al., 1998). TIMP-3 uniquely amongst the TIMPs has the ability to inhibit angiogenesis directly by competitively binding the VEGFR-2 receptor. (Qi et al., 2003)

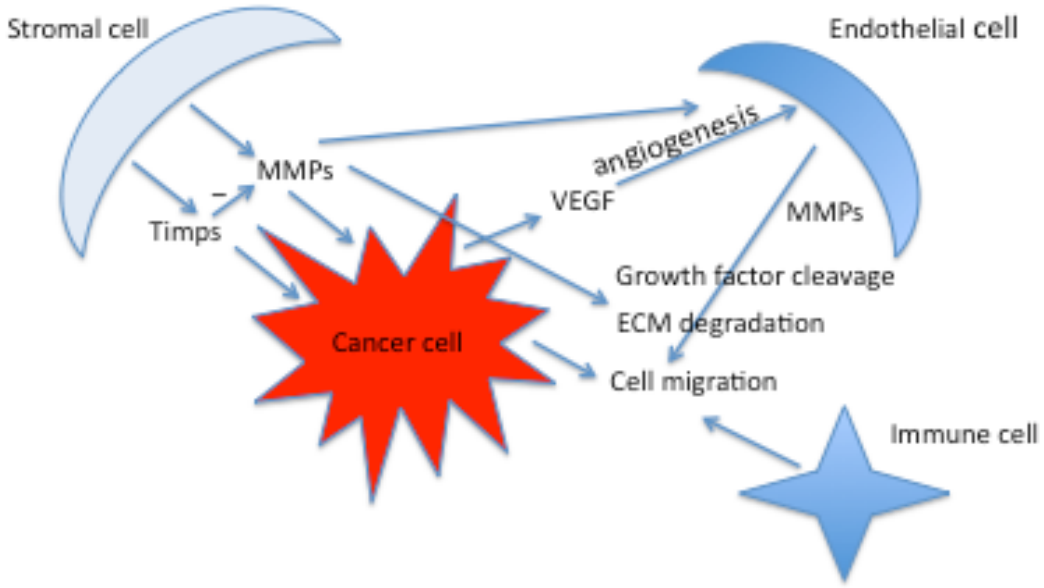


Figure 1.3 Schematic diagram showing the interplay between MMPs and Timps in cancer progression

1.4 Angiogenesis in multiple myeloma

Bone marrow angiogenesis, as measured by microvessel density (MVD), has been shown to be markedly elevated in myeloma compared to its premalignant state, monoclonal gammopathy of unknown significance (MGUS) (Vacca et al., 1994). The increase in angiogenesis was found to correlate with the plasma cell proliferative rate rather than plasma cell number. Bone marrow angiogenesis has also been shown to have prognostic value in myeloma (Rajkumar et al., 2000; Sezer et al., 2000). Vacca et al showed that overall survival in patients with newly diagnosed myeloma was significantly different among those with high, intermediate and low grade angiogenesis with median times of 2, 4 and 4.4 yrs respectively. Munshi showed a correlation between high MVD and poor event free survival, overall survival and duration of complete response in patients with myeloma treated with standard induction chemotherapy followed by tandem autotransplants and interferon maintainance (Munshi and Wilson, 2001). Similarly MVD also predicts overall survival following autologous transplantation with a median overall survival from diagnosis of 40 months for those with high grade angiogenesis compared with a significantly higher figure for those with low grade angiogenesis (Kumar et al., 2004). Interestingly angiogenesis appears to persist even after treatment with chemotherapy, autologous tranplantation or even the use of antiangiogenic agents such as

thalidomide (Kumar et al., 2002; Rajkumar et al., 1999; Singhal et al., 1999). Sezer however has shown that MVD in patients with myeloma who did achieve remission with treatment was significantly lower than pre-treatment levels (Sezer et al., 2001b). Overall these studies suggest that angiogenesis plays a part in the transformation of MGUS to myeloma and in the progression of early stage myeloma to advanced refractory disease.

As with other tumours induction of angiogenesis in myeloma is thought to be mediated by an alteration in the balance between pro and anti-angiogenic cytokines. A number of pro-angiogenic cytokines are upregulated in patients with active myeloma. VEGF is one of the key pro-angiogenic cytokines thought to play a role in myeloma pathogenesis. VEGF was shown to be overexpressed in plasma cells from myeloma patients with an increase in VEGF receptors in the surrounding stromal cells (Bellamy et al., 1999; Dankbar et al., 2000). Similarly Ribas et al demonstrated significantly increased VEGF immunostaining in bone marrow biopsies from myeloma patients which had prognostic impact on overall and event free survival on univariate analysis but not on multivariate analysis (Ribas et al., 2004). On the other hand Molina et al were unable to demonstrate increased VEGF expression in myeloma cells either by immunohistochemistry or by real time reverse transcriptase polymerase chain reaction studies. Although serum VEGF levels have not been shown to be elevated in myeloma, they are significantly elevated in patients with POEMS syndrome (Soubrier et al., 1997), a plasma cell disorder related to myeloma. Serum levels of another proangiogenic factor basic fibroblast growth factor (bFGF) were also shown to be elevated in myeloma by Sezer et al (Sezer et al., 2001a) and these levels decreased with effective chemotherapy. Matrix metalloproteinases (MMPs) play a key role in degrading the extracellular matrix surrounding tumour cells thereby facilitating the proliferation and migration of endothelial cells. Myeloma cells can not only produce pro-MMPs but also stimulate stromal cells including endothelial cells to produce MMPs. Barille et al showed that myeloma cells produce MMP-7 which in addition to its direct proteolytic activity on the extracellular matrix, also converts pro-MMP-2 produced by stromal cells to its active form (Barille et al., 1999). Myeloma cells were also shown to express MMP-2 and -9 with surrounding tumour cells secreting MMPs-1 and -2 (Barille et al., 1997).

A number of antiangiogenic therapeutic strategies have been evaluated in myeloma with varying degrees of efficacy but the results overall further confirm the role of angiogenesis in the pathogenesis of myeloma. The discovery by Barlogie's group that thalidomide, a drug with potent antiangiogenic properties, was highly effective in patients with relapsed,

refractory myeloma (Singhal et al., 1999) led to a number of other antiangiogenic compounds being developed for use in myeloma. Lenalidomide, an analogue of thalidomide, has shown greater efficacy than thalidomide in myeloma with reduction in angiogenesis through inhibition of VEGF secretion being one of its mechanisms of action (Rao, 2007). The proteosomal inhibitor Bortezomib is now well established in the treatment of myeloma and in addition to its proteosomal inhibitory effects it has been shown to have significant inhibitory effects on endothelial cell proliferation and migration as well as to downregulate VEGF and angiopoietin expression by endothelial cells (Roccaro et al., 2006). In view of the integral part that VEGF is thought to play in tumour angiogenesis several inhibitors of the VEGF signaling pathway have been developed. Bevacizumab, a humanized monoclonal antibody against VEGF and approved for the treatment of colorectal carcinoma is now being evaluated in myeloma. Initial studies with VEGF receptor tyrosine kinase inhibitors including PTK787/ZK222584 (Novartis) and GW654652 (GlaxoSmithKline) showed some anti myeloma efficacy in vitro (Podar and Anderson, 2005). Pazopanib is a novel small molecule tyrosine kinase inhibitor of VEGF receptors -1, -2 and -3 which has shown antiangiogenic and anti-myeloma effects in a xenograft mouse model. Phase 1/11 studies using this compound in myeloma patients are ongoing (Podar et al., 2006). Bisphosphonates are another class of drugs that have proven efficacy against myeloma bone disease as well as having direct antitumour effects in myeloma. Furthermore it is now clear that they also have significant antiangiogenic effects by inhibiting VEGF and VEGFR expression (Scavelli et al., 2007).

1.5 Angiogenesis in leukaemias

There is increasing evidence now supporting a role for angiogenesis even in acute myeloid and lymphoid leukaemias. MVD was found to be raised in bone marrow biopsies from patients with acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL) and chronic myelomonocytic leukaemia (CMML) (Aguayo et al., 2000; Hussong et al., 2000). Perez-Atayade et al showed evidence of increased MVD in the bone marrow of children with acute lymphoblastic leukaemia (ALL) (Perez-Atayde et al., 1997).

Numerous preclinical studies have also demonstrated the close relationship between angiogenesis and leukaemia cell survival. Human ALL cells injected into non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice induce bone marrow neovascularisation (Veiga et al., 2006). Furthermore bone marrow endothelial cells when

exposed to plasma from patients with ALL were shown to undergo proliferation, migration and tube formation, none of which occurred following exposure to plasma from normal patients (Veiga et al., 2006). Co-culture of ALL cells with bone marrow endothelial cells significantly reduced leukaemia cell apoptosis when compared with ALL cells cultured in control conditions. These experiments strongly support the hypothesis that leukaemia cells induce angiogenesis which in turn promotes leukaemia cell survival and expansion. Further evidence supporting the biological relationship between leukaemia and angiogenesis comes from the observation that leukaemic cells home to bone marrow vascular niches mediated by stromal derived factor 1 (SDF-1) and its receptor CXCR4 (Sipkins et al., 2005). SDF-1 is highly expressed by endothelial cells in these bone marrow vascular niches and CXCR4 is highly expressed on ALL cells.

Fiedler et al showed that in most patients with AML, VEGF is expressed by leukaemic blasts (Fiedler et al., 1997). Cellular VEGF levels were shown to be higher in AML than in normal bone marrow and found to have prognostic significance (Aguayo et al., 1999). There was a correlation between higher VEGF levels and shortened overall and disease free survival. VEGF receptors have also been demonstrated on leukaemic blasts suggesting that leukaemia cell survival and/or proliferation may be maintained, atleast partly, by an autocrine VEGF pathway (Dias et al., 2000). Expression of the VEGF receptor neuropilin-1 (NRP-1) in bone marrow is significantly increased in AML patients, with high expression correlating with poor overall survival. 5yr OS rates were 16.9% in patients with high NRP-1 levels compared with 49.6% in those with low levels (Kreuter et al., 2006). Different patterns of angiogenic factor expression can be recognized between the different haematological malignancies suggesting they may play different roles depending on the type of leukaemia. For instance plasma VEGF levels were not elevated in MDS or ALL (Aguayo et al., 2000). However Koomagi et al did show elevated levels of VEGF in childhood ALL with a trend to longer relapse free and overall survival in patients with low VEGF levels (Koomagi et al., 2001). Bellamy et al studied a panel of haematopoietic cell lines and found about 50% expressed bFGF compared with 100% that expressed VEGF (Bellamy et al., 1999). Elevated plasma and urinary levels of bFGF were detected in urine from patients with ALL and elevated bFGF levels correlated with increased bone marrow vasulature (Perez-Atayde et al., 1997). Interestingly bFGF levels failed to significantly diminish with clinical remission.

As with myeloma, expression of the metalloproteinases MMP-2 and -9 is upregulated in both adult and childhood ALL with MMP-2 positivity correlating with the presence of

extramedullary disease in adult ALL (Kuittinen et al., 2001). MMP-2 expression of ALL cell lines correlated with the ability to invade matrigel in vitro and with the capacity to invade and metastasize in a SCID mouse model (Hendrix et al., 1992). MMP-9 expression in lymphoblastic cell lines was also found to be important for invasion and metastasis in Matrigel assays (Ivanoff et al., 1999). The role of MMP-2 and -9 in AML is more complex with variable expression levels of MMP-2 and -9 being seen in the conditioned media of myeloid cell lines and in blasts from AML patients (Janowska-Wieczorek et al., 1999). In vitro Matrigel assays show that invasion of myeloid cells through Matrigel can be inhibited by the natural metalloproteinase inhibitor TIMP-2 and by a blocking antibody against MMP-2 but not by an anti MMP-9 antibody suggesting that myeloid cell invasion was largely dependent on MMP-2 activity (Sawicki et al., 1998). In a study by Kuittinen et al MMP-2 expression strongly correlated with good prognosis in AML patients (3yr EFS = 82% for MMP-2 positive patients compared with median EFS of 13.5 months for MMP-2 negative patients) (Kuittinen et al., 1999). In contrast Lin et al found significantly lower plasma MMP-9 levels in patients with AML compared with normal controls and this also correlated with improved overall survival (>12 months in those with low levels compared with 4 months when MMP-9 levels were high) (Lin et al., 2002). MMP-9 levels were no different to controls when patients were in complete remission but levels were shown to drop again at the time of relapse.

A number of different antiangiogenic molecules have been evaluated in leukaemia patients with mixed results. Thalidomide when used in combination with other chemotherapeutic agents has shown only modest efficacy in AML with complete response rates of upto 25% (Barr et al., 2007; Steins et al., 2003). Clinical trials with single agent Bevacizumab in patients with relapsed/refractory AML showed a reduction in bone marrow VEGF expression but no significant antileukaemia effect (Zahiragic et al., 2007). A phase II trial of Bevacizumab used in combination with cytarabine and mitoxantrone has shown encouraging results (ORR 48%, CR 33%, PR 14.5%) in patients with relapsed/refractory AML with a reduction in serum VEGF levels and bone marrow MVD following treatment (Karp et al., 2004). Several small molecule tyrosine kinase inhibitors of the VEGF and PDGF pathways have also been used to treat patients with AML. SU5416 (inhibitor of VEGFR-1,-2, c-kit and Flt-3 receptors) when used on its own to treat patients with refractory AML resulted in an overall response rate of 19% (Fiedler et al., 2003). PTK787 (inhibitor of VEGFR-1,-2,-3, PDGFR and c-kit receptors) failed to demonstrate efficacy when used as monotherapy to treat relapsed AML but in

combination with cytarabine and daunorubicin resulted in a complete remission rate of 29% (5 out of 17 patients) (Roboz et al., 2006).

1.6 Angiogenesis in lymphomas

MVD is increased in low and high grade non Hodgkins lymphomas compared with benign lymphadenopathies (Ribatti et al., 2000). Increased MVD has also been shown in involved organs from patients with adult T cell leukaemia/lymphoma compared to normal controls (Kchour et al., 2008). Jorgenson et al reported on a large study of non Hodgkins lymphoma patients including peripheral T-cell (PTCL), follicular and diffuse large B-cell lymphoma and showed increased MVD in all subtypes with high interfollicular MVD scores predicting poorer overall and event free survival in follicular lymphoma (Jorgensen et al., 2007). In contrast Koster et al showed that increased MVD was associated with a favourable outcome in patients with FL receiving first line treatment (Koster et al., 2005). One possible explanation for this contradictory finding is the use of interferon-α (IFN-α) in the treatment of this patient group with those patients with a higher MVD possibly responding better to IFN-α, a potent antiangiogenic agent. Tzankov et al evaluated angiogenesis parameters in DLBCL, FL and mantle cell lymphoma and found that the aggressive lymphomas had the highest MVD and VEGF expression but failed to demonstrate any correlation with clinical outcome (Tzankov et al., 2007). Similarly Hazar et al failed to show any prognostic significance for MVD in patients with NHL (Hazar et al., 2003). Passalidou et al argued that the phenotype of angiogenesis was more important than MVD when comparing high grade and low grade lymphomas with more immature vasculature in DLBCL compared with a more mature vascular phenotype in FL (Passalidou et al., 2003). In a smaller study on HL, Mainou-Fowler showed increased MVD in lymph node biopsies along with increased VEGF expression (Mainou-Fowler et al., 2006). Further proof of the role of angiogenesis in HL comes from Glimelius et al who have shown significantly worse progression free survival (PFS) in patients with high MVD compared with those with low MVD (Glimelius et al., 2005). In a large study of 286 patients with HL, MVD was found to be an independent adverse prognostic factor in relation to disease free survival (Korkolopoulou et al., 2005).

In Jorgenson's study of NHL patients VEGF expression was also found to be increased, by immunohistochemistry, in all subtypes with the highest expression seen in PTCL. The pattern of VEGF expression (diffuse versus focal) was found to have prognostic significance with diffuse expression associated with worse overall survival in FL. VEGF

expression has also been shown to correlate with tumour grade - DLBCL patients show higher VEGF expression in comparision with low grade lymphoma (Ho et al., 2002). High serum VEGF levels were shown to correlate with poor prognosis in non Hodgkins lymphoma (Salven et al., 1997). Passam also showed increased serum VEGF expression in untreated patients with non Hodgkins lymphoma compared to normal controls with no significant decrease in levels even with successful treatment (Passam et al., 2008). Bertolini et al in a study of NHL patients showed an inverse correlation between serum VEGF levels and event free survival (Bertolini et al., 1999). Significantly increased VEGF expression has also been demonstrated in Hodgkins lymphoma, angioimmunoblastic lymphoma and Castleman's disease either by immunohistochemistry (Doussis-Anagnostopoulou et al., 2002), insitu hybridisation (Foss et al., 1997) or serum ELISA (Rueda et al., 2007). Evaluation of preclinical lymphoma models have revealed a correlation between the extent of VEGF production by lymphoma cell lines in vitro and the efficiency of tumour engraftment in immunodeficient mice (Fusetti et al., 2000).

Serum levels of bFGF were shown to be elevated in patients with NHL and high pretreatment levels were found to be associated with poor overall survival (5 yr OS of 39% in high bFGF expressors compared with 60% in low expressors) (Salven et al., 1999). In this study multivariate analysis showed that bFGF had a stronger prognostic value than serum lactate dehydrogenase. These findings were corroborated by Pazgal et al who showed that patients with NHL who expressed bFGF had a significantly worse progression free and overall survival than those who did not[96]. In addition those patients expressing the bFGF receptor-1 were significantly less likely to achieve a complete remission than those lacking the receptor (33% v 67%, p=0.047)

Diffuse large B-cell lymphoma (DLBCL) patients with high levels of tissue MMP-9 were found to have significantly worse overall survival than those patients who did not express MMP-9 (Sakata et al., 2004). In follicular lymphoma, MMP-2 and MMP-9 expression was found to correlate with higher grade disease with MMP-9 positivity predictive of good chemotherapy response although it failed to have prognostic significance (Pennanen et al., 2008). In Hodgkins lymphoma, MMP-9 was expressed by Reed Sternberg cells but again this did not correlate with overall survival (Flavell et al., 2000).

Circulating endothelial progenitor cells were found to correlate with tumour volume in a lymphoma xenograft model (Monestiroli et al., 2001). Igreja et al showed that circulating EPCs were seen more frequently in lymphoma patients than in healthy controls, with

higher numbers found in younger patients and in those with aggressive lymphomas (Igreja et al., 2007). Levels of EPCs declined with successful treatment of the lymphoma.

Bevacizumab has shown modest clinical activity in NHL when used as a single agent or in combination with R-CHOP chemotherapy (Ganjoo et al., 2006). There was also a marginal correlation between baseline VEGF levels and response to treatment in this study. Antisense oligonucleotides against VEGF have also been used to treat lymphoma patients, with a reduction in plasma VEGF levels after treatment and limited efficacy (Levine et al., 2006). Thalidomide as well as its analogue lenalidomide have shown significant activity against indolent lymphomas like mantle cell lymphoma when used as monotherapy or in combination with chemotherapy (Kaufmann et al., 2004; Smith et al., 2008). Low dose 'metronomic' therapy appears to target the tumour vasculature rather than directly inhibiting tumour proliferation and this approach has been shown to be successful in DLBCL (Buckstein et al., 2006). In this study where celecoxib and low dose cyclophosphamide were used to treat relapsed DLBCL patients, clinical response was seen in 37% of patients and this correlated with declining levels of EPCs. Sunitinib (a broad inhibitor of VEGFR-1,2 and 3 and PDGF receptors) has shown promise in lymphomas and is currently being evaluated in phase II trials for DLBCL (Buckstein et al., 2007).

1.7 Antiangiogenic therapy for haematological malignancies

With significant evidence emerging to support a critical role for angiogenesis in the pathogenesis of haematological malignancies, there has been increasing interest in developing antiangiogenic therapeutic strategies for these disorders. There are several theoretical reasons why targeting the neovasculature may be advantageous. Delivery of anti-cancer drugs to tumour cells is often impaired because of abnormalities in blood flow arising from altered tumour blood vessel architecture and function. In contrast to tumour cells, the endothelial cells lining the tumour blood vessels are more readily accessible to therapy. Furthermore endothelial cells unlike tumour cells are homogenous and genetically stable and less likely to develop mutations causing resistance to drugs (Kerbel, 1991). Also the same drug can potentially be used to treat a range of malignanices when endothelial cells rather than the tumour cells are the target.

As highlighted earlier angiogenesis is a complex process with multiple, sequential and interdependent steps. Four broad categories of antiangiogenic drugs can be described based on which step of the angiogenesis process is the target for inhibition. These include

(1) inhibitors of endothelial cell activation, (2) inhibitors of endothelial cell proliferation and migration, (3) drugs that inhibit the degradation of the extracellular matrix and (4) inhibitors of the integrin receptors. The majority of drugs that inhibit endothelial cell activation target the VEGF pathway – either as monoclonal antibodies binding VEGF (Bevacizumab) or the VEGFR-2 receptor (DC101) or as small molecule tyrosine kinase inhibitors of the VEGR receptors which include SU5416 (blocks VEGFR-2), SU6668 (blocks VEGF, bFGF and PDGF receptors) and ZD4190 (blocks VEGFR-1 and VEGFR-2 receptors). All of these drugs have shown efficacy in preclinical tumour models and are currently in phase II and phase III clinical trials. The second category of antiangiogenic drugs inhibits the proliferation and migration of endothelial cells. They include TNP-470, thalidomide, angiostatin and endostatin amongst others. Thalidomide is a potent antiangiogenic agent that targets multiple angiogenic pathways. When used as a single agent in myeloma it produces a partial response (50% paraprotein reduction) in atleast 30% of patients (Singhal et al., 1999). In combination with dexamethasone it results in a response rate of 63% compared with 41% when given dexamethasone alone (Rajkumar et al., 2006). Thalidomide in combination with Rituximab results in a complete response rate of 31% in relapsed/refractory mantle cell lymphoma (Kaufmann et al., 2004). Similarly it has been shown to be effective when combined with fludarabine treatment in CLL, with overall response rates of 80% for previously untreated and 25% for relapsed patients (Giannopoulos et al., 2009). Several drugs that inhibit degradation of the extracellular matrix (MMP inhibitors) have been evaluated in a number of tumours including myeloma with limited success (Vihinen and Kahari, 2002). The reasons for this include the lack of selectivity of the MMP inhibitors, insufficient knowledge of which MMPs play the most crucial role in tumourigenesis, the use of these drugs in end stage disease where angiogenesis may be less important, the use of single agent therapy as opposed to adjuvant therapy and dose limiting toxicity. The final group of antiangiogenic drugs include inhibitors of the integrins, which play an important role in activating numerous signaling pathways that promote endothelial cell proliferation and migration. Several inhibitors of integrins are currently in clinical trials for example alpha4 integrin inhibitors in myeloma.

Drug	Target	Study entities	Approved for
Receptor tyrosine kinase inhibitors			
PTK787/ZK 222584 (Vatalanib®)	VEGFR1-3, PDGFRβ, c-Kit	AML, PMF, MDS, CML, DLBCL, MM	
SU5416 (Semaxinib)	VEGFR1-2, c-kit, Flt3	AML, MDS, MM, MPN	
Sorafenib (Nexavar®)	VEGFR2-3, B-Raf, Faf-1, PDGFRβ	AML, ALL, MDS, CML, CLL, NHL, MM	Advanced renal cell carcinoma, HCC
Sunitinib (Sutent®)	VEGFR1-3, PDGFRα+β, c-kit, Flt3	AML, MDS, CLL, Myeloma, NHL	Advanced renal cell carcinoma, GIST
PKC-412 (Midostaurin)	VEGFR2, PKC, PDGFR, Flt3, c-Kit	AML	
Cediranib (Recentin®)	VEGFR1-3, PDGFRβ, c-Kit	AML, MDS, CLL	
Proteasome inhibitors			
Bortezomib (Velcade®)	26S proteasome, NF-κB	AML, ALL, MDS, CML, NHL, MCL	MM, MCL
Anti-VEGF strategies			
Bevacizumab (Avastin®)	VEGF-A	AML, MDS, CLL, CML, NHL, MM	Metastatic colorectal cancer, NSCLC, breast cancer
Immunomodulatory drugs			
Thalidomide	bFGF, VEGF, IL-6	AML, MDS, MPN, CLL, NHL, MM	MM
Lenalidomide (Revlimid®)	bFGF, VEGF, IL-6	AML, MDS, CLL, NHL	MM, 5q- MDS

Table 1.1 Selection of ongoing clinical trials with antiangiogenic therapies for haematological malignancies

(adapted from (Medinger and Mross, 2010))

1.8 Interferons

Interferons (IFNs) are a family of cytokines with multiple biological effects including antiangiogenesis. Other key actions include inhibition of tumour cell proliferation, immunomodulation and regulation of antiviral responses. Two major classes of interferons have been described – types I and II based on their ability to bind to distinct receptor types. The common type I interferons include IFN-α, IFN-β, IFN-ω and IFN-τ,

all of which bind to the type I IFN receptor. IFN-γ is the sole type II IFN and binds to a distinct type II receptor.

1.8.1 Apoptotic effects of Interferons

The antitumour effects of IFNs also result from their ability to induce apoptosis in tumour cells as well as endothelial cells. The primary mechanism of IFN induced apoptosis is thought to be activation of the caspase pathway. IFNs upregulate death receptor ligands such as TNF related apoptosis inducing ligand (TRAIL) and Fas which bind to their respective receptors thereby activating Fas-associated death domain protein (FADD) which in turn activates caspase-8 leading to activation of the caspase cascade (Thyrell et al., 2002). It has also been postulated that caspase-9 can be independently activated through the release of cytochrome c triggered by IFN mediated mitochondrial activation (Thyrell et al., 2002). Other pathways shown to be involved in IFN induced apoptosis include increased activity of serine-threonine protein phosphatase 2A (Saydam et al., 2003) and upregulation of TNF-α (Baker et al., 2002). Induction of apoptosis by IFNs is cell type dependent and the magnitude of the response can vary between the different IFN species. For instance IFN-β has a greater pro-apoptotic effect than IFN-α or IFN-γ against melanoma, ovarian carcinoma and multiple myeloma cell lines (Chawla-Sarkar et al., 2001; Chen et al., 2001; Morrison et al., 2001). Resistance to the apoptotic effects of IFNs can arise from tumour mediated inhibition of pro-apoptotic IFN stimulated genes such as XAF-1 (X-linked inhibitor of apoptosis protein (XIAP) associated factor-1) or overexpression of inhibitory proteins like XIAP.

1.8.2 Antiproliferative activity of Interferons

IFNs induce a number of different genes some of which have been shown to regulate cell proliferation such as 2'5' oligoadenylate synthetase (2-5A) and dsRNA-dependent protein kinase (PKR) (Lengyel, 1993). In addition, extracellular signal-regulated kinase (ERK) and mitogen-activated ERK- regulating kinase (MEK) play a critical role in regulating cell proliferation through their effects on the cell cycle by promoting degradation of mitotic inhibitors ($p21^{WafI}$ and $p27^{KipI}$) and downregulating the cyclin-dependent kinases (Cdk -2 and -4). Type I IFNs have been shown to inhibit the MEK-ERK pathway resulting in impairment of cell cycle progression and ultimately a reduction in cell proliferation (Romerio and Zella, 2002). Other mechanisms that contribute to the

antiproliferative activity of IFN include increased cell rigidity, depletion of essential metabolites and suppression of proto-oncogenes (Marth et al., 1992).

The antiproliferative effects of IFNs against malignancies have been demonstrated in a wide variety of solid tumour cell lines in vitro (Borden et al., 1982). With respect to haematological malignanices, IFNs are known to inhibit the proliferation of AML, ALL (Lee et al., 2010), myeloma (Einhorn et al., 1988) and lymphoma cells (Leandersson and Lundgren, 1980) in both primary cells and cell lines.

1.8.3 Immunomodulatory function of Interferons

IFNs have long been known to have profound immunomodulatory activity involving both innate and adaptive immune responses. Type I IFNs are responsible for activating macrophages, natural killer cells and cytotoxic T cells as well as stimulating a Th1 T helper response (Goodbourn et al., 2000). They also have anti-inflammatory properties by inhibiting production of tumour necrosis factor-α, interleukin-8 and stimulating production of interleukin-10.

1.8.4 Antiangiogenic effects of Interferons

There is now considerable evidence demonstrating that IFNs have significant antiangiogenic activity either by their direct inhibitory effects on endothelial cells or indirectly by downregulating tumour derived pro-angiogenic cytokines. Several investigators have shown that type I and II IFNs inhibit proliferation of endothelial cells in vitro (Feldman et al., 1988; Minischetti et al., 2000; Ruszczak et al., 1990). Others however have challenged this view by reporting increased proliferation of endothelial cells after IFN-α treatment (Cozzolino et al., 1993; Gomez and Reich, 2003). Pammer et al postulated that the antiangiogenic activity of IFN-α resulted from endothelial cells undergoing replicative senescence, with this phenomenon only being seen after prolonged exposure to IFN-α (Pammer et al., 2006). Sgonc et al have demonstrated increased apoptosis of ECs in response to IFN-α (Sgonc et al., 1998). In addition IFN-α and β inhibit endothelial cell migration and invasion as well as formation of capillary-like structures in Matrigel assays (Albini et al., 2000).

IFNs also inhibit angiogenesis indirectly by downregulating the production of pro-angiogenic cytokines by tumour cells and stromal cells. Reduced VEGF expression in response to IFN-α and -β has been shown in hepatocellular carcinoma (Wu et al., 2005), neuroendocrine tumours (Rosewicz et al., 2004), Ewings tumour (Sanceau and

Wietzerbin, 2004), neuroblastoma (Streck et al., 2004) and peripheral blood mononuclear cells (Salven et al., 2001). Similarly IFN-α and -β downregulate expression of bFGF in bladder carcinoma (Dinney et al., 1998), colon carcinoma (Ozawa et al., 2001), renal cell carcinoma (Vermeulen et al., 1997) and neuroblastoma (Streck et al., 2004). Production of the matrix metalloproteinases MMP-2 and MMP-9 are also suppressed by type I and II IFNs in a variety of tumour cell lines (Fabra et al., 1992; Gohji et al., 1994; Kato et al., 1995).

The net result of the direct and indirect antiangiogenic effects of IFNs is inhibition of tumourigenesis. This has been demonstrated in both preclinical tumour models and in clinical trials. Experiments involving xenograft models of bladder carcinoma, neuroblastoma, prostate carcinoma and Ewings sarcoma have shown significant tumour regression in vivo when exposed to IFN-α/β along with a reduction in microvessel density. The antitumour effects of IFN were apparent when recombinant protein was administered parenterally as well as following viral mediated expression of IFN either systemically or from tumour cells. Sidky et al also showed that the antiangiogenic effects of IFN were independent of its antiproliferative effects ie, inhibition of angiogenesis did not occur solely as a result of a reduction in tumour cell mass. Experiments in a murine bladder carcinoma and murine leukaemia model indicated that the reduction in microvessel density following treatment with murine IFN-β resulted not from any antiproliferative effects against the tumour cells but most likely from direct inhibition of angiogenesis pathways (Sidky and Borden, 1987).

1.8.5 Therapeutic use of Type I Interferons in clinical trials

Type I interferons have been used in humans to treat a variety of malignancies with mixed results. IFN-α has been extensively used in renal cell carcinoma either as monotherapy (Minasian et al., 1993) or in combination with interleukin-2 (Negrier et al., 1998) and 5-Fluorouracil (Hofmockel et al., 1996) with moderate response rates and significant prolongation of event free survival. In melanoma IFN-α in combination with chemotherapy has shown increased response rates but this has not translated into improved overall survival (Jonasch and Haluska, 2001).

IFN-α has also been used to treat a variety of haematological malignancies including chronic myeloid leukaemia (CML), hairy cell leukaemia, acute myeloid leukaemia, multiple myeloma and follicular lymphoma. Until the advent of tyrosine kinase inhibitors IFN-α formed the mainstay of treatment of patients with CML who were not eligible for a

bone marrow transplant. IFN-α treatment resulted in a haematological response rate of 50-70% and improved progression free and overall survival in patients with CML (1994; Allan et al., 1995). IFN-α has also been shown to be highly effective in hairy cell leukaemia with response rates of over 80% (Quesada et al., 1984). In the UK Medical Research Council AML11 trial low dose IFN-α (3MU thrice weekly) was used as maintenance treatment in older patients with AML with no significant benefit in relapse risk or overall survival (Goldstone et al., 2001). In myeloma, induction treatment with combination chemotherapy (melphalan and prednisolone) and IFN-α was shown to result in a significantly improved response rate than with chemotherapy alone (68% versus 42% respectively) (Osterborg et al., 1993). Other studies however have failed to demonstrate a beneficial effect for IFN-α during induction treatment for myeloma (Gertz et al., 1995; Oken et al., 1999). IFN-α has also been used as maintenance treatment in myeloma and was shown to improve median duration of response and overall survival in a study by Mandelli et al (Mandelli et al., 1990). These results however have not been corroborated by other studies (Peest et al., 1995; Salmon et al., 1994). The study by Westin et al showed a prolongation of duration of response with IFN-α maintenance treatment but no difference in survival (Westin et al., 1995).

1.8.6 Antitumour activity of interferon-β

The majority of clinical studies in haematological malignancies using type I IFNs have been with IFN-α. However there has been an increasing number of preclinical and clinical studies evaluating the antitumour effects of IFN-β against a range of solid tumours as well as haematological malignancies. In vitro experiments have suggested greater antitumour effects for IFN-β compared with IFN-α against melanoma (Johns et al., 1992) and glioma (Rosenblum et al., 1990) cell lines. IFN-β was shown to have significant antitumour efficacy in xenograft models of Ewings sarcoma (Sanceau and Wietzerbin, 2004), bladder carcinoma (Izawa et al., 2002), neuroblastoma (Streck et al., 2004) and prostate carcinoma (Dong et al., 1999). In human trials IFN-β has been shown to be effective in hairy cell leukaemia with a daily dosing regimen of 2-6 $x10^6$ IU/m2 (Liberati et al., 1990b; Liberati et al., 1991). Phase II trials with IFN-β in myeloma showed only a minimal response with transient stabilisation of disease followed by tumour progression once treatment was discontinued (Liberati et al., 1990a). Combination treatment using recombinant interleukin-2 (IL-2) and IFN-β proved no more effective than IL-2 alone when treating patients with non-Hodgkin's lymphoma and significant side effects were

seen (Duggan et al., 1992). At the outset of this thesis no other group had evaluated the use of IFN-β in patients with AML.

1.8.7 Limitations in the clinical use of IFNs-α/β

A major limiting factor in the therapeutic use of IFNs is their toxicity. In many of the above mentioned studies there was a significant discontinuation rate due to side-effects of fever and flu-like symptoms. Furthermore due to its short half life IFN has to be administered atleast three times a week resulting often in poor compliance. Failure to adequately maintain therapeutic levels of IFN in plasma may be a significant limiting factor in its efficacy against malignancies.

1.9 Adeno associated virus

Adeno associated virus vectors (AAV) along with oncoretroviral vectors are among the most frequently used viral vectors for gene therapy. The lack of pathogenicity of the virus, the persistence of the virus and the availability of multiple serotypes with differing tropism have been the main factors facilitating its use as a delivery vector for gene therapy.

1.9.1 AAV structure and life cycle

AAV is a single stranded DNA virus which requires the presence of a helper virus for infection. Uniquely amongst viruses, wild type AAV demonstrates site specific integration into a region on chromosome 19q13.4 termed AAVS1. The most well characterised serotype AAV-2 genome consists of single stranded DNA about 4.7kb in length. There are two 145bp inverted terminal repeats (ITRs) flanking two open reading frames (ORFs) which contain the REP and CAP genes. The ITR acts as the origin of replication and serves as a priming site for second strand synthesis by DNA polymerase. They are also essential for AAV genome packaging, transcription and site specific integration The first ORF encodes four REP proteins Rep78, Rep68, Rep52 and Rep40 which are required for DNA replication, site specific endonuclease and DNA binding activity. The second ORF encodes three viral capsid proteins – VP1, VP2 and VP3 with molecular weights of 87, 72 and 62 kda respectively (see Figure 1.4).

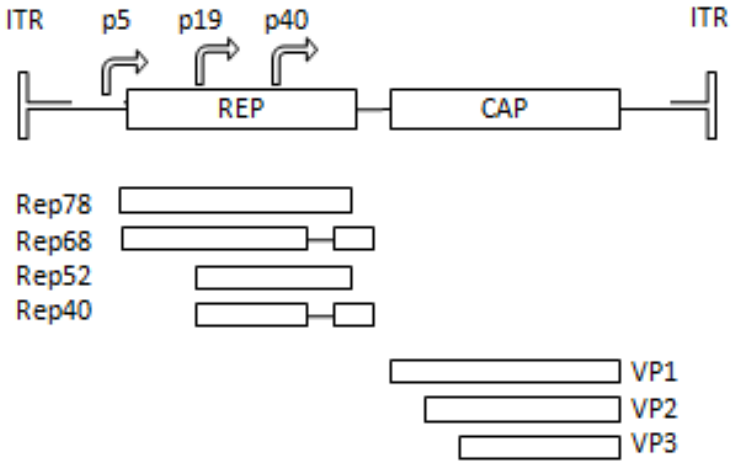


Figure 1.4 Schematic diagram of AAV genome and encoded proteins

AAV-2 gains entry into host cells by binding to the heparin sulphate proteoglycan receptor followed by internalisation mediated through interactions with several coreceptors including $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrins, fibroblast growth factor receptor 1, hepatocyte growth factor and laminin receptor. AAV virions are endocytosed into clathrin coated vesicles and traffic to the nucleus where they are released from endosomes and undergo uncoating of the viral capsids prior to nuclear entry. Once inside the nucleus AAV, in the presence of helper virus, enters a lytic stage with viral genome replication, expression and virion production. In the absence of helper virus AAV enters a latent cycle and integrates at the AAVS1 locus.

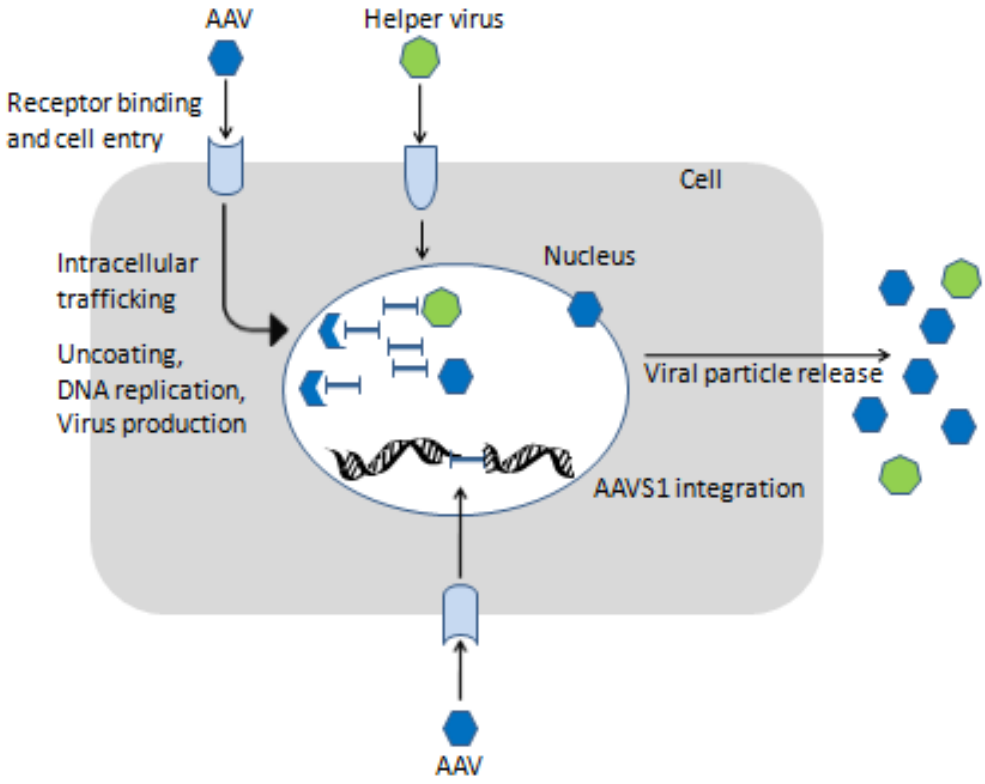


Figure 1.5 Schematic diagram showing the life cycle of wild type AAV

1.9.2 AAV as a gene therapy vector

For the purpose of gene therapy applications recombinant AAV (rAAV) vectors have been constructed lacking in REP and the integration efficiency element (IEE) required for site specific integration. This ensures that rAAV vectors persist in host cells primarily episomally reducing the potential risk of insertional mutangenesis. On the other hand the site specific integration capacity of AAV vectors into the potentially safe AAVS1 site is now being harnessed by developing mutant vectors that express REP during transduction but prevent it from being incorporated. This would enable safe integration and long term transgene expression of rAAV into stem cells where cell turnover would otherwise have resulted in loss of transgene from episomal AAV in the progeny.

Most of the early rAAV vectors were based on AAV-2 but these vectors were found to be inefficient both in terms of the time required to reach peak expression (several weeks) and the level of transgene expression in targeted tissues such as muscle, brain, liver and retina. The efficiency of AAV transduction is dependent on each of the steps of AAV infection namely AAV binding, cell entry, trafficking to the nucleus, nuclear entry, uncoating and second strand synthesis. However the rate limiting factors were found to be

inefficient AAV trafficking and second strand synthesis. One strategy adopted to reduce the lag time before optimal transgene expression and potentially enhance the level of expression was to develop self complementary (scAAV) vectors where the transgene is packaged as complementary dimers within a single virion. scAAV vectors bypass the need for second strand synthesis and have been shown to improve the transduction efficiency of the liver in mice by at least 20 fold compared with conventional AAV vectors (Nathwani et al., 2006). The packaging capacity of a scAAV vector (2.2kb) is half that of of ssAAV vectors, however this is still sufficient for a great number of applications.

Atleast 10 different AAV serotypes have now been described with differing capsid protein sequences. Although the receptors have been identified for only some of the serotypes, what is clear is that the tropism of the serotypes are very different. This property has been utilised to pseudotype rAAV vectors with the appropriate capsid depending on the intended application. For example AAV-9 serotype was found to exhibit greater tropism for the central nervous system when compared to serotypes 1,6 and 8 (Gray et al., 2011). Similarly both AAV5 and AAV-8 pseudotyped rAAV vectors show significant tropism for the liver in non human primates (Nathwani et al., 2007).

A further potential limitation of rAAV vectors is the host immune response to AAV infection. Preexisting neutralising antibodies, because of prior infection, account for the humoral response seen towards AAV. Cell mediated responses to AAV have been reported and are thought to depend on the route of administration and AAV serotype. Selective use of appropriate AAV serotypes (for instance use of AAV-8 which is less likely to encounter pre-existing immunity on account of its low seroprevalence) and the use of adjuvant immunosuppressants are strategies being developed to mitigate the host immune response.

A number of clinical trials have now been conducted with rAAV vectors with some still ongoing. Most of the trials have been for monogenic diseases where the gene product is either non functional or absent as in Haemophilia B, cystic fibrosis, Duchene muscular dystrophy and Leber congenital amaurosis. However rAAV vectors have also been used in other disorders such as Parkinson's disease, rheumatoid arthritis and prostate cancer. The most impressive results so far have been when rAAV was delivered directly into the retina to treat Leber's Congenital Amaurosis (Maguire et al., 2008) and also when used intravenously to treat Haemophilia B (Nathwani et al., 2011).

1.10 Lentiviral vectors

HIV derived lentiviral vectors have become increasingly popular in both clinical and non clinical research on account of their unique ability to stably transduce both dividing and non dividing cells making them one of the most efficient gene delivery vectors.

Lentiviruses are enveloped RNA viruses from the Retroviridae family of viruses. They are composed of 2 copies of RNA, a nuclear capsid, a capsid, a membrane associated matrix, envelope proteins such as surface glycoproteins and transmembrane proteins, enzymes such as integrase, protease, reverse transcriptase and accessory proteins namely Nef, Vif, Vpu, Vpr. The replication cycle of lentiviruses begins by first binding to a host cell surface receptor via the surface glycoprotein of the virus. The membranes of the viral envelope and the host cell surface membrane then fuse allowing entry of the virus into the cell. Following entry, uncoating and reverse transcription takes place leading to the formation of a pre-integration complex which can actively enter through the nuclear envelope into the nucleus of the infected cell and integrates into the host genome. Importantly nuclear entry by the pre-integration complex is not dependent on cell division unlike other retroviral vectors. Transcription and translation are followed by assembly of structural viral proteins with viral RNA leading to newly formed viral particles which escape from the cell by budding and mature outside the cell.

Lentiviral vectors have undergone sequential improvements to the original HIV based construct in order to minimise the risk of accidentally generating a replication competent virus and also to increase their packaging capacity as well as to improve their transduction efficiency for a wide variety of mammalian cell types. Some of the current third generation lentiviral vectors use a 4 plasmid vector system with the structural gag and pol genes, the REV gene and the env gene encoded by separate plasmids with the packaging sequence (ψ) deleted from these plasmids. Furthermore the HIV accessory proteins such as tat, vpr, vrf and nef have all been deleted. The fourth plasmid with an intact ψ sequence encodes the transgene of interest. By splitting the vector system into 4 plasmids, the number of recombination events between the plasmids required to generate a replication competent virus is extremely high and to date there have been no reported cases of such an event occurring.

Other modifications made to the lentiviral vector include a deletion in the U3 region of the 3' LTR making the LTR effectively inactive, the use of heterologous sequences such as the vesicular stomatitis virus envelope gene to replace the native HIV envelope which enhances safety as well as to broaden tropism and replacement of the U3 region of the 5'

LTR with other promoter sequences to drive tat-independent transcription. The addition of the woodchuck hepatitis virus post transcriptional regulatory element (WPRE) has been shown to stabilise transgene mRNA levels and thereby enhance transgene expression. Insulator elements such as the chicken β-globin locus HS4 element have also been used to separate transgene expression from regions of the chromosome that surround the site of vector integration thereby reducing the potential genotoxic risks of vector insertion.

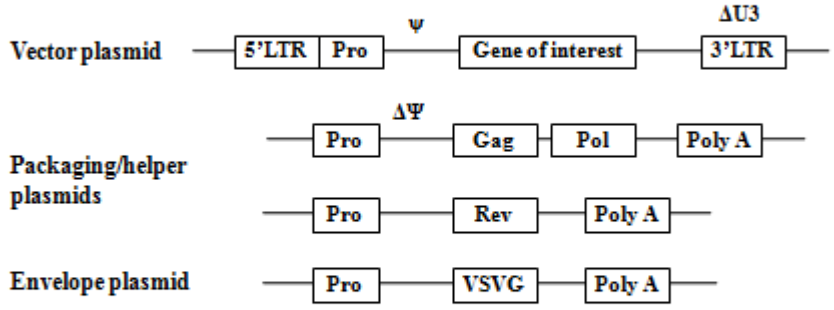


Figure 1.6 Schematic diagram showing the four plasmid lentiviral vector system

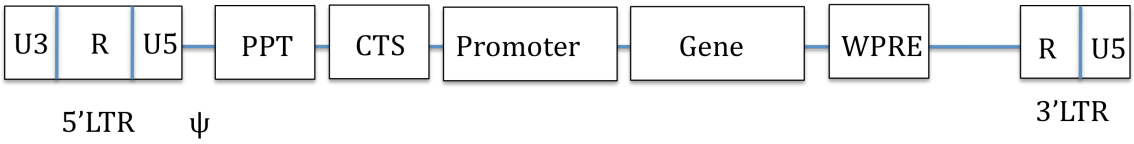


Figure 1.7 Basic structure of a third generation self inactivating lentiviral vector. CA, capsid; CTS, central termination site; LTR, long terminal repeat; PPT, polypurine tract; Ψ, packaging sequence; WPRE: woodchuck hepatitis B posttranscriptional element.

Onco-retroviral vectors have now been shown to cause insertional mutagenesis not only in mouse models but also in patients with X-linked severe combined immunodeficiency who were transplanted with haematopoietic stem cells transduced with a murine oncoretroviral vector expressing the common γ-chain of the interleukin-2 receptor (Cavazzana-Calva., et al 2000; Howe., et al 2008), some of whom developed leukaemia. In these patients it was shown that the retroviral vector integrated in proximity to the

LMO2 proto-oncogene promoter resulting in aberrant expression of the LMO2 gene and subsequent uncontrolled cell proliferation. Enhancer elements within the oncoretroviral LTR were thought to responsible for perturbation of the LMO2 gene. Lentiviral vectors were shown to be less genotoxic compared with oncoretroviral vectors in a Cdkn2a-/- mouse transplantation model which is susceptible to a broad range of cancer triggering genetic lesions (Montini., et al 2006). Furthermore there have been no oncogenic events observed in clinical trials using lentiviral vectors to date although clonal expansion and dominance of haematopoietic progenitors has been reported in a gene therapy trial of thalassaemia using haematopoietic cells transduced with a lentiviral vector expressing the β-globin gene (Cavazzana-Calvo., et al 2010).

A number of clinical trials utilising lentiviral vectors are now underway for indications such as X-SCID, ADA-SCID and Wiskott-Aldrich syndrome where haematopoietic stem cells are being transduced, HIV infection and B-cell NHL where T cells are the target cell ex vivo as well as conditions such as Stargardt's Macular degeneration and Retinitis pigmentosa where the vector is directly administered to the retina of patients.

1.11 Summary and Aims

Angiogenesis plays a key role in the pathogenesis of haematological malignancies such as acute myeloid leukaemia, multiple myeloma and lymphoma. The evidence supporting this hypothesis includes the finding of increased bone marrow microvessel density and increased levels of plasma and urinary pro-angiogenic cytokines in patients with these malignancies as well as the encouraging results from preclinical and clinical studies using antiangiogenic therapies. The common limiting factor in most of these studies has been the short half-life of many of the antiangiogenic compounds. The pharmacokinetics of these antiangiogenic molecules has necessitated the use of cumbersome administration protocols with significant side effects seen in association with peak levels. This has resulted in poor compliance and an inability to administer the drug long enough to result in its desired therapeutic effect.

Interferons are a group of cytokines with significant antitumour activity as a result of their anti-proliferative, anti-apoptotic and anti-angiogenic effects. Interferon-α has been shown to have some anti-leukaemic and anti-myeloma activity but again its short half-life has limited its therapeutic potential. Previous work has demonstrated more potent antitumour responses to IFN-β than IFN-α. The effects of IFN-β on AML however have not previously been studied in any systematic fashion.

The aim of this thesis is to investigate the therapeutic effects of stable antiangiogenic expression in xenograft models of AML, lymphoma and myeloma. More specifically the antitumour effects of blocking the vascular endothelial growth factor (VEGF) pathway and the matrix metalloproteinase pathway will be investigated by stably expressing either the soluble VEGFR-2 receptor (sFLK-1) or TIMP-3 using viral vectors. In addition we will also evaluate the effects of sustained systemic expression of hIFN-β in xenograft models of AML using AAV vectors. Furthermore the mechanisms of action of IFN-β will be investigated and its anti-tumour efficacy compared with the more commonly used cytokine IFN-α.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Cell culture

All cell lines were obtained from ATCC (www.ATCC.org).

2.1.1 Adherent cell lines

Human embryonic kidney transformed 293T cells and HeLa cervical carcinoma cells were cultured in 20cm^2 tissue culture plates in Dulbecco's Eagle's Medium (DMEM-Life Technologies) with 10% heat inactivated fetal calf serum and 1% penicillin, streptomycin and amphotericin (D10) at 37°C and 5% CO_2. Cultures were split by trypsinisation when cells approached confluence every 2-3 days. This was done by washing cells gently with PBS lacking Ca2+ or Mg2+ and then adding 3mls of warm 1x Trypsin/EDTA which results in detachment of cells within 2-5 minutes. D10 medium was added to terminate the trypsin reaction and cells were then split 1:5 into fresh tissue culture plates.

2.1.2 Suspension cell lines

The AML cell lines HL-60, KG-1α, AML193 and OCI AML5 were cultured in 80cm^2 tissue culture flasks in RPMI 1640 medium (Life Technologies) with 10% heat inactivated fetal calf serum (FCS) and 1% penicillin, streptomycin and amphotericin (R10) at 37°C and 5% CO_2. GMCSF at 100pg/ml was added to the medium for AML193 and OCI AML5 cells. Cells were maintained at a density of 1x10^6/ml by splitting the culture 1:10 every 2-3 days with fresh medium. The myeloma cell lines ARH77 and KMSBM as well as the lymphoma cell lines Raji and U937 were also cultured in a similar way without any growth factor supplements.

2.1.3 Primary leukaemia cells

Primary leukaemia cells (blasts) were obtained from patients with relapsed AML under the care of University College London Hospitals NHS Trust, in accordance with local ethical guidelines and with LREC approval. When clinically indicated, patients with high peripheral blast counts were subjected to a standard apheresis procedure and the leukaemia cells subsequently separated by Ficoll gradient centrifugation. Mononuclear cells (>90% of which were blasts) were frozen in liquid nitrogen in RPMI medium with 50% FCS and 10% dimethyl sulphoxide. Once thawed cells were cultured in RPMI medium with 10% FCS in the absence of cytokines.

2.2 Microscopy and Immunostaining

2.2.1 Routine microscopy

Cytospin preparations and blood smears were visualised following May-Grunwald-Giemsa staining with an Olympus IX70 inverted microscope. Cell counts and viability assessments were made using trypan blue exclusion and a haemocytometer.

2.2.2 Flow cytometry

An EPICS Elite flow cytometer (Beckman-Coulter) was used for both Fluoresence-activated cell sorting (FACS) analysis and cell sorting. Usually 0.5-1.0 $x10^6$ cells were stained with 5µl of the appropriate FITC, PE or APC conjugated antibody (human CD45, CD13 or CD138 – all from BD Biosciences) for 20 minutes at room temperature. Cells were then washed twice with FACS buffer (PBS with 2% FCS) and suspended in 400µl of either FACS buffer or fixative (1% paraformaldehyde). Samples were run on the flow cytometer and both percentage fluorescence and mean cell fluorescence was measured. A minimum of 10000 events was usually collected for each sample. Cell sorting was usually done with GFP by setting gates around the top 25% mean GFP +ve population using standard methods.

2.2.3 Immunohistochemistry

Organs harvested from sacrificed mice were promptly fixed in 10% formaldehyde/saline for atleast 24 hours. Bone tissue was subsequently decalcified in 10% formic acid for 48-72 hours. Fixed/decalcified tissue was then paraffin embedded and 3 µm tissue sections cut for immunohistochemical studies and mounted on Capillary Gap Microscope slides (Dako Ltd., High Wycombe, Berks, UK). Prior to immunostaining tissue sections were put through a heat mediated antigen retrieval step by placing them in a pressure cooker for 2 minutes at full pressure in 3 L of citrate buffer (pH 6.0). Sections were then stained with a primary monoclonal anti-human CD45RB antibody (Dako M0833, 1:100 dilution) for 2 hours after blocking with a mouse immunoglobulin blocking reagent (Vector labs PK-2200) for one hour. Slides were then incubated with a biotinylated goat anti-mouse antibody, stained with Streptavidin Peroxidase before final addition of a chromogenic substrate (Dako ChemMate Detection kit K5001).

2.3 Cell proliferation/apoptosis/viability assays

2.3.1 Tritiated thymidine assay

Tumour cell lines were seeded in 96 well plates at 20,000 cells per well in 200µl of RPMI with 10% FCS to which varying concentrations of recombinant human IFNβ-1a (Avonex) or IFNα-2b (Intron A) were added. Following stimulation for 48-72 hours, cells were pulsed with ^{3}H-thymidine (0.5µC per well) for 4 hours, harvested and ^{3}H-thymidine incorporation measured by scintillation counting.

2.3.2 MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay

Tumour cell lines were seeded in 96 well plates at 20,000 cells per well in 200µl of RPMI with 10% FCS to which varying concentrations of recombinant human IFNβ-1a (Avonex), IFNα-2b (Intron A) or cytarabine were added. Cells were incubated for 48-72 hours following which MTS activity was measured by the CellTiter kit (Promega, Southampton, UK) according to manufacturer's instructions.

2.3.3 Annexin V viability assay

To determine the proportion of apoptotic cells following exposure to interferons after 48-72 hours incubation, cells were pelleted at 350g for 5 minutes and then washed once in Annexin V binding buffer (140mM NaCl, 10mM HEPES pH 7.4 and 5mM $CaCl_2$) before incubation for 10 minutes on ice with 1µl Annexin V-FLUOS (Roche) in 50µl annexin V binding buffer to which 1µl of propidium iodide (50µg/ml) was added. Specific fluorescence was then determined using an EPICS Elite flow cytometer. Apoptotic cells were calculated as the proportion of cells that were Annexin V positive and Propidium iodide negative.

2.3.4 Cell cycle analysis

Following stimulation with interferons for 72 hours, tumour cells were pelleted by centrifugation and then resuspended in 1ml 70% methanol in PBS at -20°C. After a minimum of 30 minutes fixation, the cells were pelleted once more, washed once in PBS and then incubated for 30 minutes in staining solution (200µl propidium iodide stock solution and 500µl RNAase stock solution to 10mls in PBS with Ca^{2+} and Mg^{2+}) at 37°C. Samples were then analysed by flow cytometry.

2.3.5 TUNEL (TdT-mediated dUTP Nick End labeling) assay

The TUNEL assay was used to demonstrate apoptotic cells in tumours extracted from mice treated with hIFNβ. Frozen sections from subcutaneously implanted HL-60 tumors as well as formalin fixed paraffin embedded tissue sections from intravenously injected HL-60 leukemic mice were used for TUNEL staining. Frozen sections were fixed in 4% paraformaldehyde, rehydrated in PBS and permeabilised in 1% Triton-X-100, 0.1% Sodium citrate. Paraffin sections were dewaxed, rehydrated and proteinase K treated prior to permeabilisation. A fluorescein TUNEL labeling kit (Roche Cat No 11684795001) was then used to label apoptotic cells followed by counterstaining with DAPI. Slides were analysed by fluorescence microscopy.

2.4 Protein assays

2.4.1 Western Blotting

2.4.1.1 Cell lysates

Total cell lysates were made by pelleting $5x10^6$ cells by centrifugation and resuspending them in 50µl of ice cold RIPA buffer (Sigma) containing protease inhibitors (Complete protease inhibitor tablets, Roche) for 10 minutes on ice. The lysates were spun again at 20,000g for 10 minutes and the clear supernatants removed. The appropriate volume of 4x sample buffer was added to the supernatants and the lysates boiled in a 100°C hot block for 5 minutes.

Sample Buffer (4x) : 320nM Tris pH 6.8
8% SDS
4.6% DTT
40% Glycerol
1mg/ml bromophenol blue

2.4.1.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Depending on the size of the protein of interest 10% or 15% polyacrylamide gels were prepared. Lysates were loaded into the gels and electrophoresis was performed in Mighty Small Electrophoresis Units (Amersham) by applying a 200V charge across the gel for 2 hours. A prestained protein ladder (Invitrogen) was run for each gel.

Polyacrylamide running gel (10mls - sufficient for 2 gel plates)

	10%	15%
Distilled water	2.9mls	1.2mls
1M Tris pH 8.8	3.7mls	3.7mls
30% acrylamide	3.3mls	5.0mls
10% SDS	100µl	100µl
10% ammonium persulphate	75µl	75µl
Temed	9µl	9µl

Polyacrylamide stacking gel (5mls – sufficient for 2 gel plates)

Distilled water	3.5mls
1M Tris pH 6.8	0.63mls
30% acrylamide	0.84mls
10% SDS	50µl
10% APS	38µl
Temed	7.5µl

Gel running buffer (10x) : Tris 30g (250mM)
Glycine 144g (2M)
SDS 10g (1%)
Distilled water to 1L

2.4.1.3 Transfer of proteins on to nitrocellulose membrane

Following electrophoresis the gels were removed from the glass plates and transferred onto a pre wet nitrocellulose membrane (Hybond C extra, Amersham) using a semi dry transfer apparatus (NBS Biologicals) as per manufacturer's instructions.

Gel transfer buffer (10x) : Tris 30g (250mM)
Glycine 144g (2M)
Distilled water to 1L

2.4.1.4 Incubation of nitrocellulose membranes with antibodies

After transfer of proteins onto nitrocellulose membranes they were blocked with 1% skimmed milk (Marvel, Premier Brands) in PBS overnight at 4°C. They were then incubated with primary antibodies at 1 in 1000 dilution in PBS with 3% bovine serum albumin (BSA) for 2 hours at room temperature. The membranes were washed 3 times with PBS containing 0.1% Tween (PBST) and then incubated with the appropriate secondary horseradish peroxidise conjugated anti-mouse, anti-goat or anti-rabbit (Amersham Pharmacia) antibody at a dilution of 1:10000 in PBS with 3% BSA. After a further 3 washes in PBST, protein bands were visualised by chemiluminescence (ECL or ECL plus, Amersham Pharmacia).

Primary Antibodies :

Rabbit antihuman Timp-3	(Chemicon International)
Goat antimouse VEGFR2 (Flk-1)	(R&D Systems)
Rabbit Phospho-Stat1(Ser727)	(Cell Signalling Technology)
Rabbit Phospha-Jak1(Tyr1022/1023)	(Cell Signalling Technology)
Mouse anti-β tubulin	(Chemicon International)

2.4.2 Enzyme-linked immunosorbent assay (ELISA)

2.4.2.1 sFlk-1 ELISA

A 96 well ELISA plate (Nunc) was coated with 100 µl of anti sFlk-1 antibody (R&D Systems AF644) at 1 in 40 dilution in coating buffer (0.1M $NaHCO_3$ pH 10.6) and incubated overnight at 4°C. The next day the plate was washed five times with 200 µl phosphate buffered saline Containing 0.1% Tween-20 (PBST). A blocking step was then performed with 100 µl of blocking buffer (PBST with 2% BSA) added to each well and incubated for one hour at room temperature. The plate was washed again five times as before followed by addition of 100 µl of the sFlk-1 standards (recombinant mouse VEGFR-2, R&D Systems 443-KD) and serum samples at 1 in 500 dilution for one hour at room temperature. After a further 5 washes the plate was incubated for one hour at room temperature with 100 µl of biotinylated anti sFlk-1 antibody (R&D Systems BAF644) diluted at 1 in 50 in blocking buffer. The plate was washed again and 100 µl of antibiotin peroxidase (Sigma A0185) added at 1 in 30000 dilution for one hour at room temperature followed by 100 µl of peroxidase substrate (Sigma P9187). The reaction was stopped at 15 minutes with 100µl of 3M HCL and optical density of each standard/sample measured

using an Anthos 2001 ELISA plate reader at a wavelength of 492 nm. sFlk-1 concentration of each sample was then calculated from the standard curve generated.

2.4.2.2 VEGF antibody ELISA

ELISA was performed as above with recombinant human VEGF (R&D Systems 293-VE) used to coat the plates at a concentration of 100ng/ml in coating buffer. Following the blocking step and then addition of samples/standards, peroxidise conjugated anti human IgG Fc (Sigma) was added. Optical density was measured as before after addition of the peroxidise substrate and termination of the reaction with 3M HCL.

2.4.2.3 M-protein (human immunoglobulin IgG) ELISA

ELISA plates were coated with 100 µl of anti-human IgG (Biosource AH10301) at 1µg/ml in 0.1M NaHCO3 pH 10.6 overnight at 4°C. The following day plates were washed five times with PBST and blocked with 100µl per well of PBST with 2% BSA for one hour at room temperature. The plates were washed again and 100µl of hIgG standards (ICN/cappel) and serum samples were added to the wells for 2 hours at room temperature. After washing 5 times 100µl of alkaline phosphatase (ALP) conjugated goat anti-human IgG (Biosource AH10305) was added to each well and incubated for 2 hours at room temperature. The plate was washed again as before and 100µl of ALP substrate (Sigma) was added for one hour at room temperature. Once colour change of the reaction was obvious (usually at 15 minutes), optical density was measured at a wavelength of 405nm. Concentration of hIgG in the serum samples was calculated from the standard curve.

2.4.2.4 Human VEGF ELISA

Human and murine VEGF were quantified using the R&D Systems Quantikine ELISA kits (Catalog Numbers DVE00 and MMV00 respectively) as per manufacturer's instructions.

2.4.3 Interferon-α/β antiviral assay

A functional antiviral assay was used to quantitate the amount of hIFNα/β in the serum of mice either transduced with AAV-hIFNα/β or injected with recombinant IFN. This assay measures the ability of IFN in test samples to prevent lysis of a glioblastoma cell line (2D9) by the encephalomyocarditis virus (ECMV) (Meager, 2004). Briefly 2D9 cells were seeded in a microtiter plate at 50000 cells per well and incubated overnight at 37°C.

Mouse serum samples at varying dilutions as well as recombinant IFN controls were added to the wells the following day and left for 24 hours at 37°C. The next day the plate was washed and ECMV virus was added to the wells and incubated at 37°C for 24 hours. The following day the plate was washed and Naphthol blue black substrate added followed by 0.1M NaOH, prior to measurement of absorbance at 610nm using an ELISA plate reader.

2.5 Molecular Biology

2.5.1 General DNA methods

2.5.1.1 DNA agarose gel electrophoresis

A 1% agarose gel was made by dissolving either standard agarose (GibcoBRL) or low melting point (LMP) agarose in 1 x TAE buffer in a microwave oven. 1µl of ethidium bromide was added before the gel was poured into the electrophoresis tank (Horizon 58 – Life Technologies) with the lane comb in place. Once the gel was set the tank was filled with 1 x TAE buffer and the wells loaded with DNA samples (9µl of sample with 1µl of 10x loading buffer). A DNA fragment ladder was added to one of the wells to determine the size of the resolved fragments. The gel was run approximately at 50V for 1 hour. Resolved fragments were visualised using an ultraviolet box and photographed using a Polaroid camera.

10 x TAE buffer (Tris/Acetic acid/EDTA) : 28.4g Tris
11.42 mls acetic acid
20 mls 0.5M EDTA pH 8.0
Distilled water to 1L

2.5.1.2 DNA fragment gel purification

PCR products or DNA fragments digested with restriction endonucleases were run on LMP gels, visualised on an ultraviolet box and the desired band cut from the gel using a scalpel blade. The gel fragment was then purified using a commercially available kit (QIAquick gel extraction kit, Qiagen) as per manufacturer's instructions.

2.5.1.3 Restriction endonuclease digests

Restriction digests of plasmids were done either to release fragments of interest for subsequent cloning or to check the orientation of ligated fragments. Typically 5-10µl of

plasmid (1µg/µl) was digested with 1µl of restriction enzyme, 1µl of bovine serum albumin (100µg/ml) and 3µl of the appropriate 10x endonuclease buffer in a total reaction volume of 30µl made up with distilled water. Digests were performed for one hour at 37C.

2.5.1.4 Ligation of restriction endonuclease digested fragments

Digested plasmids were first treated with alkaline phosphatase (Roche, 1µl of 10mg/ml solution for 5 minutes at 37C), where necessary, to minimise self annealing. Ligation of digested fragments was performed with 1µl T4 DNA ligase (Roche), 1µl 10x buffer, 1µl vector fragment and 1-5µl of insert fragment (vector : insert DNA molar ratio of 1:3) in a total reaction volume of 10µl with distilled water. Ligation was done at 16C overnight followed by transformation of the ligated plasmids into competent bacteria.

2.5.1.5 Transformation of competent bacterial cells

DH5α *Escherichia coli* competent cells were transformed by carefully adding 5-10µl of ligation reaction to 100µl cells and incubating on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42C and returned to ice for 2 minutes. 250µl of S.O.C medium was added to the cells which were then placed in an orbital shaker at 37C for 1 hour. Cells were then streaked on ampicillin containing agar plates for overnight culture at 37C. The following day colonies were picked for screening.

2.5.1.6 Plasmid preparation techniques

Mini-preps : These were done to provide small amounts of DNA sufficient for checking the orientation of cloned fragments by restriction digestion, generating a fragment for subsequent cloning or DNA sequence analysis. Bacterial colonies were picked from agar plates with sterile pipette tips and placed into 3mls of LB containing 50µg/ml of ampicillin in a sterile Falcon tube for overnight culture at 37C in an orbital shaker. The next day mini-preps were performed using a commercial kit (QIAprep spin miniprep kit, Qiagen) as per manufacturer's instructions.

Mega-preps : These were done to generate large amounts of DNA (upto 2.5mg) for transfection purposes. 1ml of LB culture containing bacterial cells transformed with the desired plasmid was inoculated into 500mls of LB containing 50µg/ml of ampicillin in a 4L conical flask and incubated overnight at 37C in an orbital shaker. Maxi-preps were

then done using a commercial kit (QIAGEN Plasmid Mega kit, Qiagen) as per manufacturer's instructions.

2.5.2 RT-PCR / PCR

RNA was extracted from IFN treated cell lines using Trizol (Invitrogen) as per manufacturer's instructions. Reverse transcriptase reaction was performed with 1μg of RNA using reagents from Promega (A5000) as per manufacturer's protocol in a 20μL reaction. 2μl of cDNA was then used in a 20 μl PCR reaction. Primers (from Invitrogen) directed against human VEGF, bFGF, MMP-2 and MMP-9 were used in separate PCR reactions, again using reagents from Promega (C1141). PCR was also done to amplify human FIX in DNA samples extracted from murine tissue. GAPDH or murine β-actin was used as a control gene for human tumour cell lines or murine tissue respectively. Annealing temperatures of 59°C were generally used with 30 cycles of amplification.

Primer sequences (5'-3')

VEGF	F: TCGGGCCTCCGAAACCATGA
VEGF	R: CCTGGTGAGAGATCTGGTTC
MMP-2	F: GGCCCTGTCACTCCTGAGAT
MMP-2	R: GGCATCCAGGTTATCGGGGA
MMP-9	F: CAACATCACCTATTGGATCC
MMP-9	R: CGGGTGTAGAGTCTCTCGCT
hFIX	F: GATCATGGCAGAATCACCAG
hFIX	R: GCATCTTCTCCACCAACAAC
mβ-actin	F: TGACGGGGTCACCCACACTGTGCCCATCTA
mβ-actin	R: CTAGAAGCATTTGCGGTGGACGATGGAGGG
GAPDH	F: CCC CAC ACA CAT GCA CTT ACC
GAPDH	R: CCT ACT CCC AGG GCT TTG ATT

2.5.3 Southern Blotting

DNA digest : 10μg of genomic DNA samples along with a negative control and negative DNA spiked with dilutions of standards was digested with 50 Units of restriction enzyme in a 70μl total reaction volume for 6 hours. Following digestion 10μl of 6x loading dye was added and 70μl of the mixture was loaded on a 0.8% agarose gel in 1x TBE.

Electrophoresis was carried out at 60-90V for 5-6 hours or until the orange marker dye reaches the bottom of the gel.

Denaturation of gel : The gel was stained with 10µl of 100mg/ml ethidium bromide for 10 minutes and an image of the bands was taken on an ultraviolet box. Depurination of the gel was carried out with 0.1M HCl for 30 minutes. After rinsing, the gel was denatured twice for 15 minutes in 0.5M NaOH on a shaker.

Transfer on to membrane : Denatured gel was transferred onto a nitrocellulose membrane using the TURBOBLOTTER system (Schleicher & Schuell) as per manufacturer's instructions.

Preparation of probe : The green fluoresecent protein (GFP) gene located in the lentiviral vector plasmids MSCV-Flk-1-ires-GFP, MSCV-Timp-3-ires-GFP or MSCV-ires-GFP was used as a probe. A 778bp GFP fragment was isolated from the pCAGG MCS-EGFP plasmid by EcoR1 and Not1 restriction digestion and dissolved in Tris-EDTA buffer at a concentration such that 25-30ng can be added to the reaction in a volume of no more than 34µl. The DNA probe was denatured at 95°C for 3 minutes and immediately then put on ice. Radiolabelling of the probe was carried out using a commercial kit (NEBlot Kit, New England BioLabs) and α-^{32}P dCTP as per manufacturer's instructions.

Hybridisation of membrane : The membrane, post transfer, was prewet in 2x SSC, dried on blotting paper and then crosslinked using ultraviolet light. Pre-hybridisation of the membrane was carried out with 10ml of pre-warmed hybridisation buffer (Amersham) at 42°C for atleast one hour. The radiolabelled probe was diluted in 10mls of hybridisation buffer and this was used to replace the prehybridisation buffer. The membrane was incubated with the probe overnight at 42°C.

Washing and Imaging : The following day the membrane was washed twice with 500ml of wash solution1 for 20 minutes at 65°C followed by twice with wash solution 2 again for 20 minutes at 65°C.

Wash solution 1 : 20x SSC
10% SDS 50ml
Distilled water 850ml

Wash solution 2 : 20x SSC
10% SDS 50ml
Distilled water 925ml

2.5.4 Slot Blot analysis

Preparation of sample : 50µl of purified AAV vector was DNase I treated (to remove free plasmids from prep) in a 200µl reaction with the appropriate buffer and sterile water at 37°C for 1 hour. DNase I was inactivated by incubating sample with 2µl of 0.5M EDTA at 70°C for 30 minutes. The sample was then mixed with 2µl of 10% SDS, 4µl of 10mg/ml Proteinase K, 1µl of 10mg/ml glycogen and incubated at 37°C for 2 hours. This step should break down the AAV particles and release the genome. Following this a standard phenol/chloroform DNA extraction and ethanol precipitation method was used with the final DNA pellet dissolved in 200µl AAV denaturing buffer. Simultaneously the AAV DNA standards were prepared by adding 190µl denaturing buffer to 10µl of each thawed standard. Denatured samples and standards were then transferred onto a nylon membrane through a slot blot apparatus.

Preparation of probe : DNA probes against human FIX or human IFNβ were made depending on which AAV vector prep was being quantitated. 25ng of template DNA was denatured at 95°C for 5 minutes and radioactively labelled with ^{32}P dCTP using the NEBlot kit (New England Biolabs N1500L) as per manufacturer's instructions. The radiolabelled probe (final reaction volume 100µl in TE buffer) was purified using NICK spin columns (GE Healthcare). 50µl salmon sperm DNA was added to the purified probe followed by incubation with 300µl of 0.4M NaOH at room temperature for 15 minutes. The reaction was neutralised with 0.4M HCL before proceeding to the hybridisation step.
Membrane transfer : The denatured samples and standards (200µl per sample) were transferred onto Hybond N+ membrane (GE Healthcare), prewet in 2xSSC buffer (20xSSC buffer, Sigma) using the Millipore slot blot apparatus as per manufacturer's instructions. Following DNA transfer the membrane was crosslinked under ultraviolet light for 5 minutes.

Hybridisation : The transferred membrane was initially prehybridised for one hour with 10ml of hybrisation solution (Ambion) prewarmed to 42°C. Following this the prehybridisation solution was replaced with 10mls of fresh hybridisation solution and the radiolabelled probe was added to this and incubated overnight at 42°C in a hybridisation incubator.

Membrane washing and imaging : The following day the membrane was washed twice with 500mls of wash solution I for 20 minutes in an incubation shaker at 65°C at 65rpm. This was followed by a further two washes with 500ml of wash solution II again for 20 minutes. The membrane was sealed in Saran Wrap before exposure to a phosphoimager (Typhoon FLA 9000, GE Healthcare). The titres of the vector preps were calculated from the standard curve generated.

Wash Solution I	20x SSC	100ml
	10% SDS	50 ml
	Water	850ml
Wash Solution II	20x SSC	25ml
	10% SDS	50ml
	Water	925ml

2.6 Manufacture of viral vectors

2.6.1 Lentiviral vector production

A four plasmid calcium/phosphate transient transfection method was used to generate lentivirus from 293T cells. $3x10^6$ 293T cells were seeded on $10cm^2$ tissue culture plates in DMEM medium with 10% fetal calf serum and 1% penicillin, streptomycin and amphotericin (D10). A transfection mixture was made up in water to a final volume of 500µl per plate with the following plasmids at the given concentration and 50 µl of 2.5 mM calcium.

pCL10.1 MSCV-Flk-1-ires-GFP or pCL10.1 MSCV-ires-GFP or pCL10.1 MSCV-Timp-3-GFP	(Vector plasmids)	10µg per plate
pKGP3R	(Packaging plasmid)	10µg per plate
pRTR2	(Helper plasmid)	2.5µg per plate
pCAGG VSVG	(Envelope plasmid)	2.0µg per plate

500µl of 2xHepes Buffer was added to the transfection mixture dropwise whilst vortexing followed immediately by 9ml of warm D10 medium. All volumes were multiplied by the number of plates being transfected. 10 mls of the final transfection mixture was added to each plate having aspirated out the old medium. Transfected cells were incubated at 37°C and 5% CO_2 for 16-18 hours and then observed under ultraviolet light for green fluorescence. Plates were then carefully washed twice with PBS and fresh D10 medium added. The following day medium containing virus was harvested from each plate, centrifuged at 1500 rpm (400G) for 5 minutes, filtered through a 0.2 micron sterile filter and subsequently stored in small aliquots at -70°C.

2.6.2 Lentiviral vector concentration

Lentiviral vector supernatants were concentrated by ultracentrifugation using a Beckmann ultracentrifuge. Virus was aliquoted into 30ml sterile Beckmann centrifuge tubes, sealed and centrifuged at 27000rpm (77000G) for 90 minutes in a prechilled rotor at 4°C. Following centrifugation the supernatants were discarded and the viral pellets resuspended in 300 µl of serum free medium (X-vivo 10, BioWhittaker). Concentrated virus was then stored at -70°C.

2.6.3 Lentiviral vector titration

Titration of lentiviral vector preps was performed by assessing their infectivity on HeLa cells. 50000 Hela cells per well were seeded into a 6 well plate the day before transduction. The following day medium from each well was replaced by 2 mls of a transduction mixture containing varying amounts of viral supernatant (ranging from 10µl to 1000µl) and corresponding volumes of fresh D10 medium as well as 4µg/ml of polybrene. After 48 hours HeLa cells were harvested following the addition of trypsin and subsequent inactivation with D10 medium. Cells were pelleted in FACS tubes, fixed in 0.5% paraformaldehyde and FACS analysis performed to determine the number of cells expressing GFP. The titre of each viral prep was calculated based on the number of GFP positive cells and the volume of lentiviral prep used assuming that one viral particle was required to transduce one cell.

2.6.4 AAV vector production - rAAV2, rAAV5 and rAAV8

Subconfluent 293T cells plated on 15-cm plates were cotransfected with either hFIX, hIFNβ or sFlk-1 vector plasmids, a helper plasmid encoding the adenoviral helper genes (XX6) necessary for AAV production and packaging plasmids XX2, chimeric AAV2 Rep-5cap or AAV2 Rep-8cap to generate rAAV2, rAAV5 or rAAV8 pseudotyped vector particles respectively using the calcium phosphate method. Cells were harvested between 50 to 60 hours after transfection and lysed in 1x TD buffer by freeze thawing. The crude lysate was then treated with 0.5% deoxycholate (Fisher Scientific) and 50 U/mL Benzonase (Sigma), for 30 minutes at 37°C followed by centrifugation at 6000g for 20minutes. The supernatants were filtered through a 0.45micron filter before proceeding to the purification step. rAAV-2 and -5 vectors were purified by heparin and mucin affinity chromotography respectively as previously described (Auricchio et al., 2001; Clark et al., 1999). rAAV-8 vectors were purified by ion exchange column chromatography using a method developed in house (Davidoff et al., 2004). Standard slot-blot analysis was used to determine the vector particle titer.

Transfection mixture (for 40 15cm plates)	
Vector plasmid (pAV FIX/hIFNβ/sFlk-1)	600μg
Helper plasmid (XX6)	1800μg
Packaging plasmid (XX2 or 2/5 or 2/8)	600μg
2.5M Calcium	5mls
2xHBS buffer	50mls
Water	make up to 50mls

Neutralise transfection reaction with 100mls DMEM medium with 10% FCS and then add 5mls per plate

1x TD buffer	NaCL	140mM
	KCL	5mM
	K_2HPO_4	0.7mM
	$MgCl_2$	3.5mM
	Tris	25mM

2.7 Animal studies

All animal studies were performed in accordance with the Animals Scientific Procedures Act (1986) and under the authority of the UK Home Office Project and Personal Licences. B6 SCID and $\beta 2m^{null}$ NOD/SCID mice were purchased from Charles Rivers Laboratory, UK and were housed under positive pressure in individually ventilated cages (Tecniplast, Northamptonshire, UK).

2.7.1 Collection of mouse plasma/serum

Prewarmed mice were bled from their tail vein by making an incision with a sterile scalpel blade and collecting upto 200µl of blood into either a 1.5ml eppendorf tube (for serum) or an EDTA containing paediatric blood tube (for plasma). No more than 10% of blood volume was collected every two weeks as per institutional guidelines. Collected blood samples were then spun at 8000rpm (5000G) for 15mts in a microcentrifuge and the clear supernatants (plasma or serum) harvested and frozen at -20°C.

2.7.2 Tail vein injections

A 30G needle (BD, Oxford, UK) was used to inject tumour cells or AAV virus or recombinant hIFN-β into the tail veins of prewarmed mice. A maximum volume of 300µl was injected each time making sure the inoculate was at room temperature prior to injection.

2.7.3 Intraperitoneal injections

The intraperitoneal route was used to inject recombinant hIFN-β or VEGF antibody or anti-RhD antibody. A maximum volume of 300µl was admistered using a 30G needle.

2.7.4 Subcutaneous injections

The subcutaneous route was used for administration of tumour cells to create orthotopic tumour models. HL-60 tumour cells were resuspended in up to 250µl of RPMI with 10%FCS and implanted subcutaneously in the flank regions using a 30G needle. Gowth of the tumours was monitored by visual inspection and measurement of size using callipers.

2.7.5 Analysis of murine tissues for presence of tumour

Mice were sacrificed at the appropriate endpoint as per protocol and tissues harvested for FACS analysis and immunohistochemistry. After macroscopic examination of the animal for visible signs of tumour and measurement of the spleen weight, the long bones (2 femurs and 2 tibias), liver and spleen were harvested. Half of each organ was preserved in 10% formaldehyde for immunohistochemistry while half of each spleen and two bones were used to prepare single cell suspensions for flow cytometry. Bone marrow (BM) suspensions were prepared from two femurs and one tibia. BM was flushed out with RPMI and 10% FCS using a 5 mL syringe attached to a 26-gauge needle and put through a 40μM cell strainer (BD, Oxford, UK) and collected into a 50 mL polypropylene tube. The spleen was cut up and eased through a 40μM cell strainer using the plunger of a sterile 1 mL syringe, flushed through with RPMI and 10% FCS and collected into a 50 mL polypropylene tube . The cell suspensions were pelleted at 1500 rpm (400G) for 10 minutes. The supernatant was removed and the cell pellet was re-suspended. Red cell lysis was performed with 2mL red cell lysis buffer (155mM NH_4Cl, 20mM $NaHCO_3$, 1mM EDTA; BDH, Poole, UK) for 4 minutes on ice. The cells were pelleted at 1500 rpm (400G) for 10 minutes and washed once in 10 mL of RPMI /10% FCS. Cell counts, viability and immnostaining were performed.

2.7.6 Analysis of murine tissue for AAV vector biodistribution

AAV8 transduced mice were sacrificed, organs harvested and snap frozen in liquid nitrogen. 50 mg of each organ was dissected and homogenised in liquid nitrogen using a sterile mortar and pestle. Genomic DNA was then extracted using a commercial kit (DNeasy Blood & Tissue kit 69506, Qiagen). After DNA quantitation samples were used for human FIX and murine β-actin PCR (for details see section 2.5.2).

2.7.7 Statistical analysis

Where appropriate statistical comparisions were made to look for significant differences between groups of data. Results are usually expressed as the mean +/- standard error of the mean (SEM). The paired Student t test and the Mann Whitney U test were employed using Graphpad Prism version 3 software (Graphpad, San Diego). For animal survival analysis Kaplan Meier curves were plotted and the log rank test used to analyse significance. A p value < 0.05 was considered to be statistically significant.

CHAPTER 3 : DEVELOPMENT OF MURINE MODELS FOR ACUTE MYELOID LEUKAEMIA, NON-HODGKIN'S LYMPHOMA AND MULTIPLE MYELOMA

3.1 Introduction

The purpose of this thesis was to investigate the role of angiogenesis in haematological malignancies such as acute myeloid leukaemia (AML), multiple myeloma (MM) and Non Hodgkins lymphoma (NHL) and to evaluate the effect of inhibiting angiogenesis on tumour progression. The antitumour effects of inhibiting angiogenesis are best explored in vivo using murine models of haematological malignancies. Murine models allow one to mimic the human phenotype of tumours either by implanting human tumour cell lines into immunodeficient mice (xenografts) or by using transgenic models where key tumour related genes are either deleted, mutated or overexpressed. Although transgenic murine models for AML, MM and NHL exist they are limited by poor reproducibility, failure to resemble the heterogenous phenotype of human tumours seen in patients and the relative inability to evaluate therapeutic strategies. Several xenograft models have therefore been developed by injecting both tumour cell lines and primary tumour cells into different strains of immunocompromised mice. In this chapter we discuss the tumour models chosen for our experiments. Some of the models have previously been described whilst some were either adapted or developed in our laboratory to be more suitable for our research goals.

We chose two different types of immunodeficient mice to evaluate tumour engraftment – the B6.CB17-Prkdc^{scid} strain (B6-SCID) and the β2m^{tm1Unc}NOD-Prkdc^{scid} strain (β2m^{null} NOD/SCID). Both these strains lack functional B and T cells due to defects in immunoglobulin and T cell receptor gene rearrangement. In addition β2m^{null} NOD/SCID mice are MHC class I deficient, complement deficient and have virtually no NK cells. At the outset of our work most of the previously described models had used the B6-SCID and NOD/SCID strains for human tumour engraftment with very limited information available on the β2m^{null} NOD/SCID strain. We therefore compared the B6-SCID and β2m^{null} NOD/SCID strains by performing engraftment experiments using AML, MM and NHL cell lines as well as primary cells with a view to identifying the most appropriate strain to use for our tumour models.

3.2 Acute myeloid leukaemia xenograft models

The HL-60 AML cell line was previously shown to engraft with varying degrees of success in NOD/SCID mice (Dias et al., 2001). We used this cell line and injected $1x10^7$ cells into the tail vein of B6-SCID and β2m^{null} NOD/SCID mice with or without prior sublethal irradiation. Mice were bled on alternate weeks to assess the presence of

circulating leukaemic cells and the time taken to develop hind limb paralysis, secondary to tumour infiltration, was assessed. As shown in Table 3.1 almost all $\beta 2m^{null}$ NOD/SCID mice developed leukaemia in contrast with B6-SCID mice, none of whom showed evidence of leukaemia even at 12 weeks. Irradiation did not appear to make any difference to tumour engraftment. Circulating blasts were usually identified at three weeks following tumour injection, by human CD45 (hCD45) FACS analysis (Figure 3.1) and by H&E staining of peripheral blood smears (Figure 3.2). The median time to develop hind limb paralysis was 37 days at which point mice were culled. Post mortem analysis of these mice revealed significant leukaemic infiltration in bone marrow (Figure 3.3), spleen and liver (Figure 3.4) and to a lesser degree in lung, peritoneum and paraspinal tissue (Figure 3.5). Since our eventual aim was to evaluate antiangiogenic strategies we stained for murine vasculature using an anti-murine CD31 antibody. Clear evidence of increased tumour associated microvasculature could be demonstrated both qualitatively (Figure 3.6) and quantitatively (Figure 3.7).

Table 3.1 Engraftment of haematological tumour cell lines in immunodeficient mice

Cell line injected	Mouse strain	Proportion with tumours	Median time to hind limb paralysis (days)
HL-60	β_2m^{null} NOD/SCID	7/8*	37
	B6-SCID	0/4	n/a
Raji	β_2m^{null} NOD/SCID	8/8	18
	B6-SCID	3/4	35
U937	β_2m^{null} NOD/SCID	11/12	23
	B6-SCID	3/4	45
ARH77	β_2m^{null} NOD/SCID	7/8	37
	B6-SCID	2/4	38

* 1/8 mice died early from radiation related toxicity

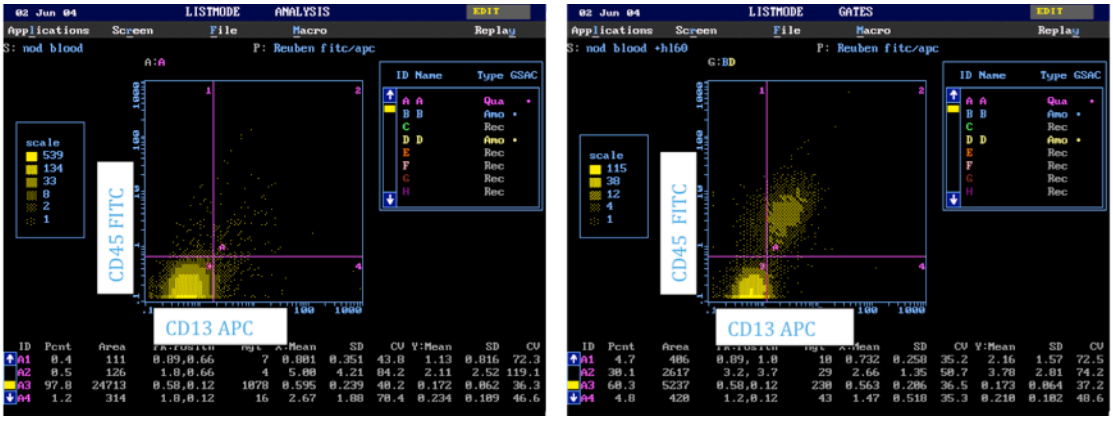


Figure 3.1 Flow cytometry demonstrating leukaemic cells (hCD13+ve/hCD45+ve) in peripheral blood of HL-60 injected β2m^{null} NOD/SCID mouse (right). Control uninjected mouse blood (left). 50µl of whole blood was stained with anti-human CD13 and CD45 antibodies followed by red cell lysis, washing and then analysis on a flow cytometer

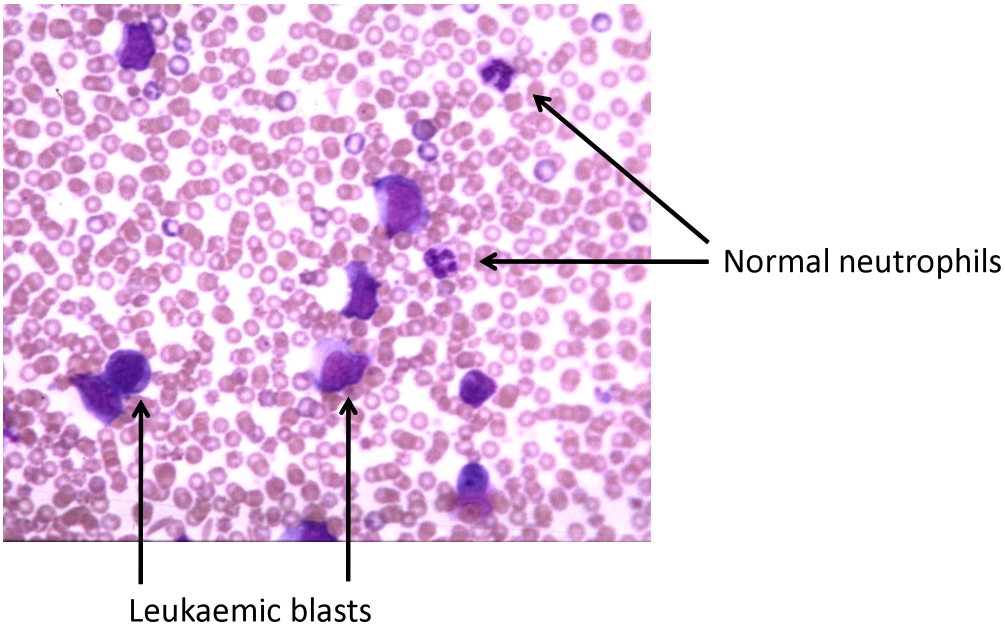


Figure 3.2 Blood film from a HL-60 injected mouse showing circulating leukaemia blasts interspersed with normal neutrophils (Magnification x40). Circulating blast percentage was calculated by counting a total of 100 white blood cells at low magnification.

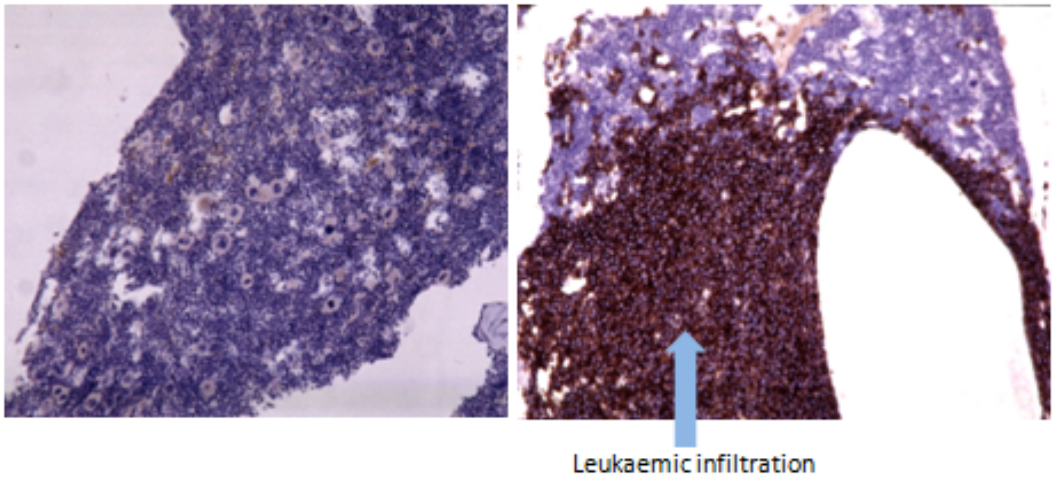


Figure 3.3 Human CD45 immunohistochemistry - bone marrow of HL-60 engrafted mouse (right) showing almost complete effacement of the marrow by hCD45 positive leukaemic cells. A normal control mouse bone marrow is shown on the left displaying normal architecture and absence of hCD45 +ve cells (Magnification x20)

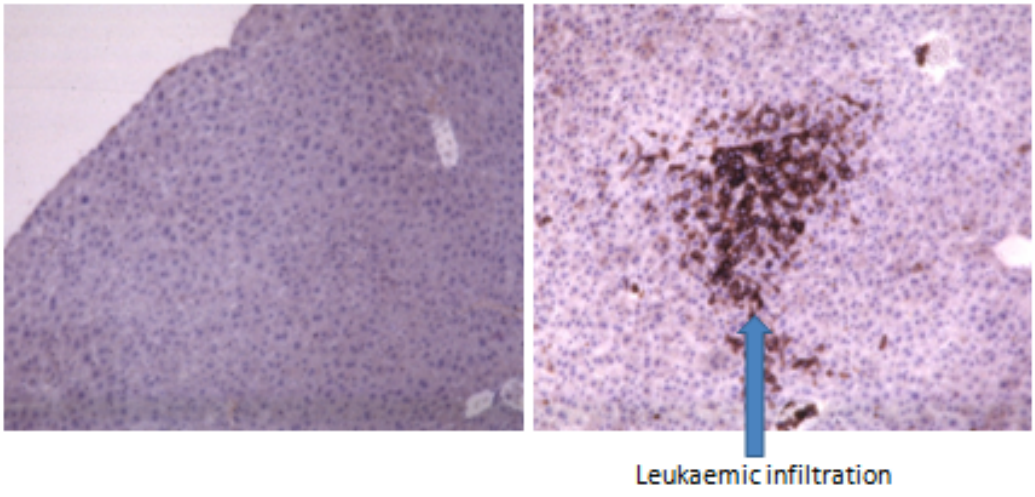


Figure 3.4 Human CD45 immunohistochemistry – section through liver from a HL-60 engrafted mouse (right) showing islands of hCD45+ve leukaemic cells in contrast to control uninjected mouse liver (left) (Magnification x20)

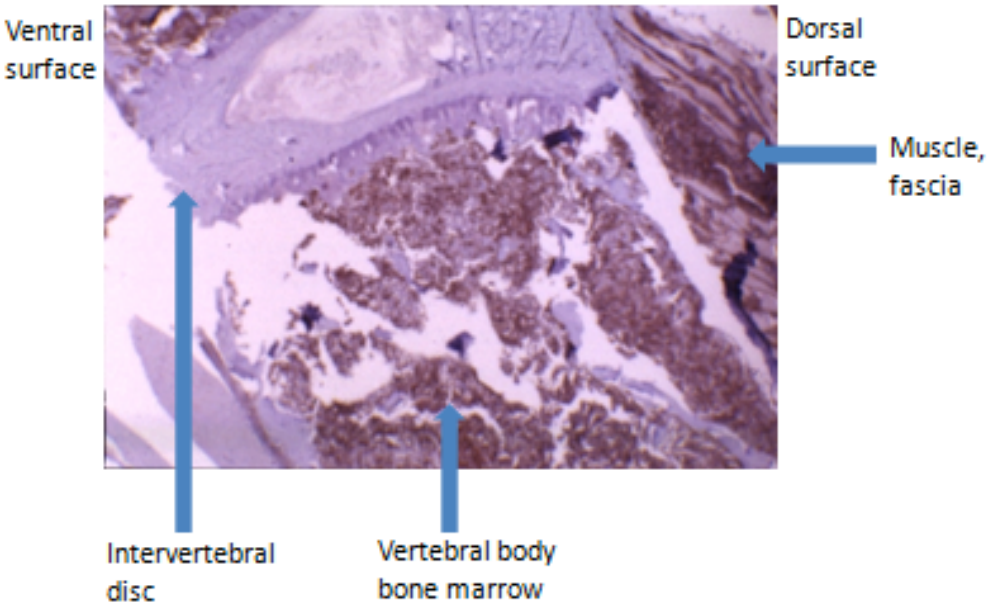


Figure 3.5 Human CD45 immunohistochemistry – sagittal section of lumbar spine from a HL-60 engrafted mouse, taken at the onset of hind limb paralysis, showing vertebral body bone marrow as well as paraspinal tissue leukaemic infiltration (Magnification x20)

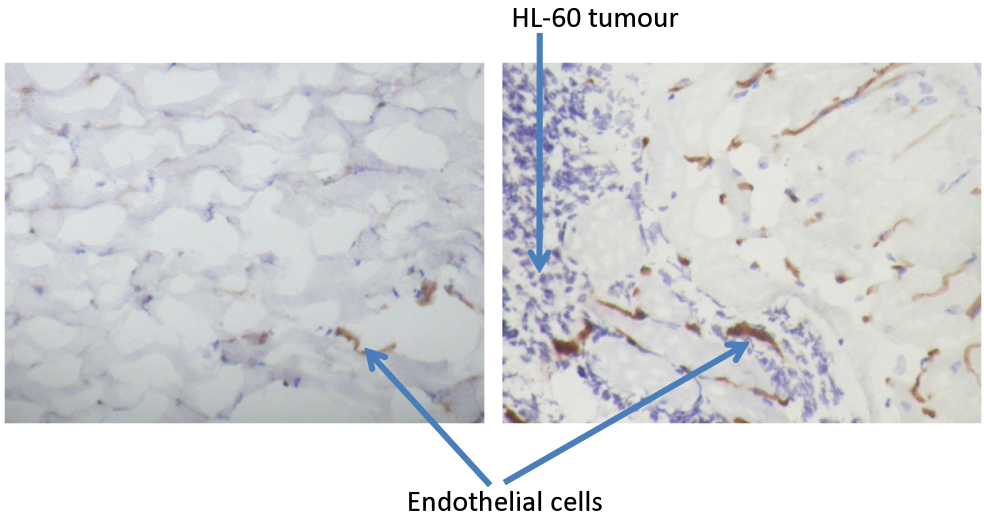


Figure 3.6 Sections of paraspinal tissue showing increased neovasculature in a leukaemic mouse (right) compared to a control β2m^{null} NOD/SCID mouse (left) using murine CD31 staining (Magnification x10)

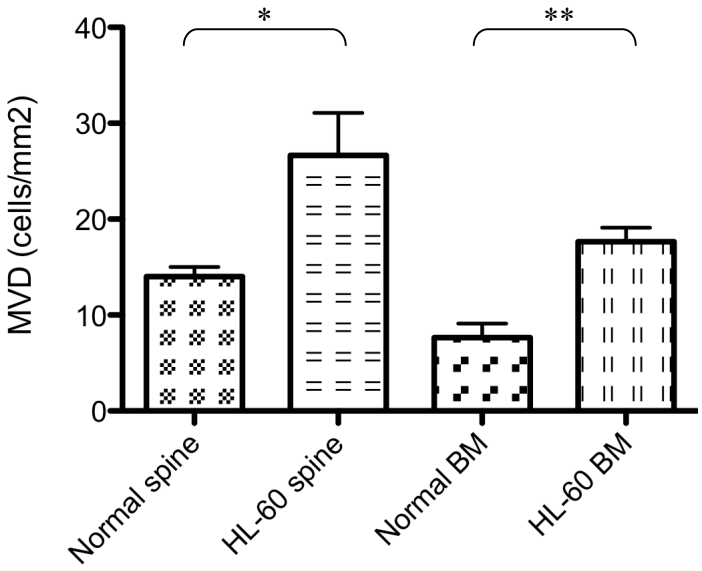


Figure 3.7 Microvessel density comparision between normal β2m^{null} NOD/SCID mice and HL-60 engrafted mice. Hot spots with increased endothelial cells were identified close to the tumour and in the case of normal mice - equivalent areas on normal tissue. Microvessel density (cells/mm^2) was quantified by two independent individuals by counting the number of CD31 positive endothelial cells in 10 high power fields within the hot spots. Significant differences in MVD in bone marrow and lumbar spine tissue were observed between tumour and normal mice (* p=0.006, **p=0.001 Mann Whitney U test)

Having established that β2m^{null} NOD/SCID mice could be used consistently to engraft HL-60 AML cells, we went on to evaluate the engraftment of primary AML cells in this particular strain. 1x10^7 primary AML cells from three different patients with poor risk AML (high white count with Flt3-ITD mutation) were injected intravenously into β2m^{null} NOD/SCID mice. Mice were monitored for signs of distress and eventually culled at 6 weeks. No circulating blasts could be identified but immunohistochemical analysis of organs on post mortem showed clear evidence of AML in bone marrow in all of the innoculated mice (Figure 3.8) (Table 3.2). We have thus been able to demonstrate reliable engraftment of HL-60 AML cells as well as primary AML cells in β2m^{null} NOD/SCID mice and have also been able to show evidence of increased tumour associated angiogenesis.

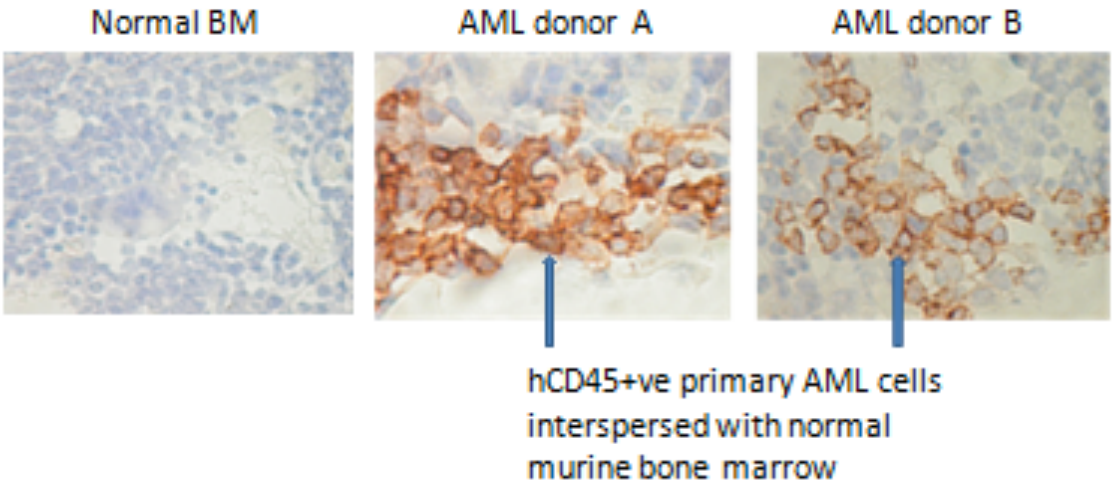


Figure 3.8 Immunohistochemistry of murine bone marrow at 6 weeks post AML injection demonstrating human CD45+ve primary AML infiltration from 2 different donors. Control BM from a saline injected β2m^{null} NOD/SCID mouse (shown on the left) stained similarly with anti-hCD45 antibody (Magnification x20)

Table 3.2 Engraftment of primary AML cells in β2m^{null} NOD/SCID mice

	Patient characteristics	Proportion of mice with AML engraftment
Patient 1	AML M4 Flt3-ITD +ve WBC 308	3/3
Patient 2	AML M1 Flt3-ITD +ve WBC 150	5/5
Patient 3	AML M1 Flt3-ITD +ve	3/4*

* ¼ mice died early from radiation related toxicity

3.3 Lymphoma xenograft models

The Raji (Burkitts lymphoma) and U937 (histiocytic lymphoma) cell lines were used in an attempt to develop a xenograft lymphoma model. As before, we initially injected intravenously $1x10^7$ lymphoma cells into both B6-SCID and $\beta2m^{null}$ NOD/SCID mice. Mice were monitored for signs of distress and development of hind limb paralysis. Our experiments showed that although the majority of mice from both strains eventually developed hind limb paralysis from disease infiltration, the time of onset was markedly different between the two strains. As summarised in Table 3.1 the median time to hind limb paralysis in the U937 model was 23 days for $\beta2m^{null}$ NOD/SCID mice and 45 days for B6-SCID mice whereas in the Raji model the median times were 18 and 35 days respectively. Immunohistochemistry with anti hCD45 antibody showed widespread Raji cell infiltration in most organs with bone marrow, spleen and liver most severely affected (data not shown), in comparision to the U937 model where tumours were seen predominantly within the abdominal cavity fixed to the peritoneum (Figure 3.9) (Table 3.3). We were thus able to prove that both B6-SCID and $\beta2m^{null}$ NOD/SCID strains could be used to engraft lymphoma cells.

Figure 3.9 Abdominal tumour from a U937 cell line injected $\beta2m^{null}$ NOD/SCID mouse

Table 3.3 Biodistribution of disease in xenograft models of haematological neoplasms

	HL-60	ARH77	KMSBM	Raji	U937
Bone marrow	+++	+++	+++	+++	+
Blood	+++	-	-	+	-
Spleen	+++	+++	-	+++	+
Liver	+++	+++	+	+++	+
Lymph nodes	+++	+	-	+++	++
Lungs	++	+	-	++	-
Kidneys	+	+	-	+	-
Peritoneum	+++	++	-	+++	+++

+++ >75% tumour infiltration by immunohistochemistry or FACS or H@E staining, ++ 25-75% tumour infiltration, + <25% tumour infiltration, - no evidence of tumour

One of the major differences between the B6-SCID and β2m^{null} NOD/SCID strains is said to be the relative lack of NK cells in the latter and this may explain its greater susceptibility to tumour engraftment. We tested this hypothesis by harvesting spleen cells from both strains of mice and staining for NK cells. As shown in Figure 3.10 FACS analysis confirms the presence of greater numbers of NK cells in the B6-SCID strain.

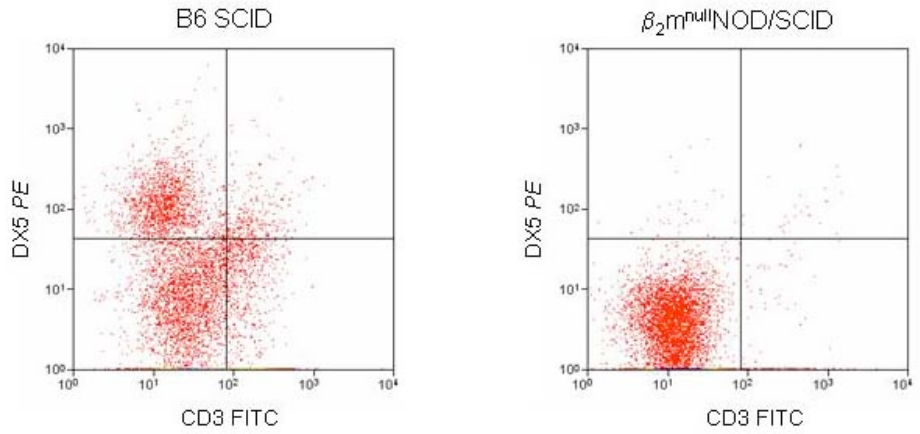


Figure 3.10 FACS plot illustrating the presence of CD3-ve DX5+ve NK cells in B6 SCID mice in contrast with the β2m^{null} NOD/SCID strain. Representative FACS plots from spleen cells of a single mouse from each strain. Atleast 5 mice from each strain were analysed similarly.

3.4 Multiple myeloma xenograft models

The ARH77 cell line, derived from a patient with plasma cell leukaemia, was originally used to develop a xenograft myeloma model by systemic injection into C.B-17 SCID mice (Huang *et al*, 1993). We have adapted this model by using B6-SCID and β2m^{null} NOD/SCID mice instead. Our results show a near 100% engraftment rate with β2m^{null} NOD/SCID mice but only 50% of the B6-SCID mice appeared to engraft regardless of prior irradiation. Median time to onset of hind limb paralysis was similar in both groups (37 and 38 days respectively). Serum human immunoglobulin was detected by ELISA and levels were shown to increase between 2 and 4 weeks following tumour injection (Figure 3.11). Immunohistochemistry with anti hCD45 antibody showed widespread tumour infiltration in most organs (Table 3.3). We were unable to demonstrate hypercalcaemia or evidence of lytic bone disease by plain X-rays in our model even though these are pathognomonic features of myeloma in humans.

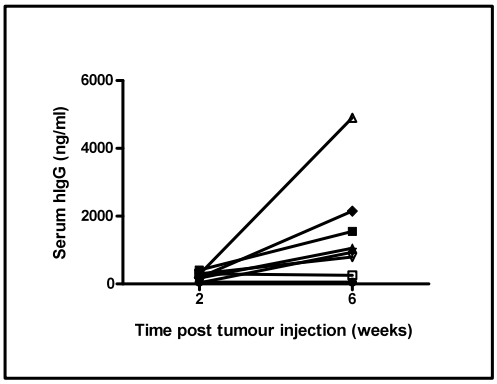


Figure 3.11 Serum human immunoglobulin levels measured by ELISA in a cohort of eight β2m^{null} NOD/SCID mice at 2 and 6 weeks following ARH77 tumour injection.

The limitations of the ARH77 model are firstly the significant extramedullary nature of disease infiltration which is in contrast to the phenotype usually seen in patients and secondly the fact that ARH77 cells have now been shown not to be bonafide myeloma cells, being EBV transformed. A new xenograft model of myeloma with predominantly medullary disease was needed and the KMS12BM model was therefore developed jointly with Neil Rabin in our laboratory (Rabin et al., 2007). The KMS12BM cell line was originally derived from a patient with non secretory myeloma (Ohtsuki et al., 1989). It was shown that intravenous injection of 1x10^7 KMS12BM cells into β2m^{null} NOD/SCID mice resulted in reliable engraftment of tumour cells predominantly in bone marrow.

Immunohistochemical analysis of organs with anti-hVS38c antibody showed evidence of tumour as early as three weeks following tumour injection. Hind limb paralysis occured at approximately 6 weeks with complete effacement of bone marrow by tumour at this stage. Imaging by micro computed tomography (microCT) showed clear evidence of bone disease in lumbar vertebrae and tibia of diseased animals (Rabin et al., 2007). My role in the development of this model extended to the tail vein injections, monitoring of the animals for paralysis, culling of the mice at the end points and harvesting and processing of the organs.

3.5 Discussion

We have described the development of xenograft models for acute myeloid leukaemia multiple myeloma and lymphoma by implantation of a number of well characterised tumour cell lines into B6-SCID and $\beta 2m^{null}$ NOD/SCID immunodeficient mice. We have shown that the $\beta 2m^{null}$ NOD/SCID strain was superior to the B6-SCID strain for engraftment of most tumour cell lines and have also confirmed that a relative lack of NK cells in the $\beta 2m^{null}$ NOD/SCID strain was one possible explanation for this.

In our HL-60 AML model we were able to track disease progression easily by analysing peripheral blood for circulating leukaemic blasts using FACS analysis, making it an ideal model for evaluation of therapeutic strategies. Importantly we were able to show engraftment of primary AML cells in the $\beta 2m^{null}$ NOD/SCID strain with disease confined to the bone marrow thus mimicking the phenotype observed in humans. We were able to develop two different types of lymphoma models (Raji and U937) – either with a rapidly growing tumour phenotype in $\beta 2m^{null}$ NOD/SCID mice or with a more slow growing tumour phenotype in B6-SCID mice. The benefit of the more rapidly growing tumour is the ability to assess response to therapy more quickly whilst it may be argued that response to antiangiogenic strategies would be better seen in the more slow growing tumour model. With our ARH77 myeloma model, disease progression can be monitored by measuring serum human immunoglobulin levels enabling us to assess potential therapeutic responses in a relatively non-invasive manner. The KMSBM model, with a much slower rate of tumour growth, is potentially more suitable for assessment of bone disease and the antitumour efficacy of antiangiogenic strategies.

CHAPTER 4 : VIRAL VECTOR MEDIATED GENE TRANSFER

4.1 Introduction

To evaluate the effects of inhibiting angiogenesis on tumour progression we decided to adopt a gene transfer approach using viral vectors to over express antiangiogenic genes either within the tumour cell or in the microenvironment of the tumour cell. Two different viral vector systems were used for our experiments - lentiviral and adeno-associated viral (AAV) vectors. The advantages of using lentiviral vectors include their high transduction efficiency, their ability to stably transduce non dividing cells and their large packaging capacity. This makes them ideal vectors to use for transduction of haematopoietic stem cells as well as most tumour cells with large and potentially multiple antiangiogenic genes. The disadvantages include the potential safety aspects of an HIV based vector and the lack of a suitable lentiviral packaging cell line with the consequent need to perform labour intensive transient transfection to produce virus. Recombinant AAV (rAAV) vectors have been shown to stably integrate into both dividing and non dividing cells, mediate long term expression and have low immunogenicity. Their limitations include the low packaging capacity, a variable transduction efficiency depending on the target cell and the development of neutralising antibodies to the viral capsid in vivo. AAV vectors based on serotype 2 (AAV2) were the first to be characterised and are the most widely used. However a number of alternative serotypes such as AAV5 and AAV8 have now been developed with potential advantages over AAV2. In this chapter the production, purification and functional assessment of our lentiviral and rAAV vectors encoding antiangiogenic genes will be described.

4.2 Lentiviral vectors

4.2.1 Cloning of sFlk-1-GFP, TIMP-3-GFP and IR-GFP lentiviral constructs

Bicistronic lentiviral vectors encoding sFLK-1 or TIMP-3 along with GFP were previously cloned by Dr. Nathwani's group (Figure 4.1). In addition a control vector expressing GFP alone was also constructed. The 8.7kbp control pCL10.1 MSCV ires-GFP construct was cloned by ligating the 1.6kbp fragment obtained by BstE11/Not1 restriction digest of the retroviral MSCV-ires-GFP plasmid with the 7kbp fragment obtained by BstE11/Not1 digest of pCL10.1 MSCV-GFP plasmid. Similarly the 11kbp pCL10.1 MSCV Flk-1-ires-GFP was constructed by ligating fragments from BstE11/Not1 digests of MSCV-Flk-1-ires-GFP and pCL10.1 MSCV Timp-3 plasmids. Finally the 9.4kbp pCL10.1 MSCV Timp3-ires-GFP construct was cloned by ligating

fragments from BstE11/Btr1 digests of MSCV Timp-3-ires-GFP and pCL10.1 MSCV Flk-ires-GFP plasmids.

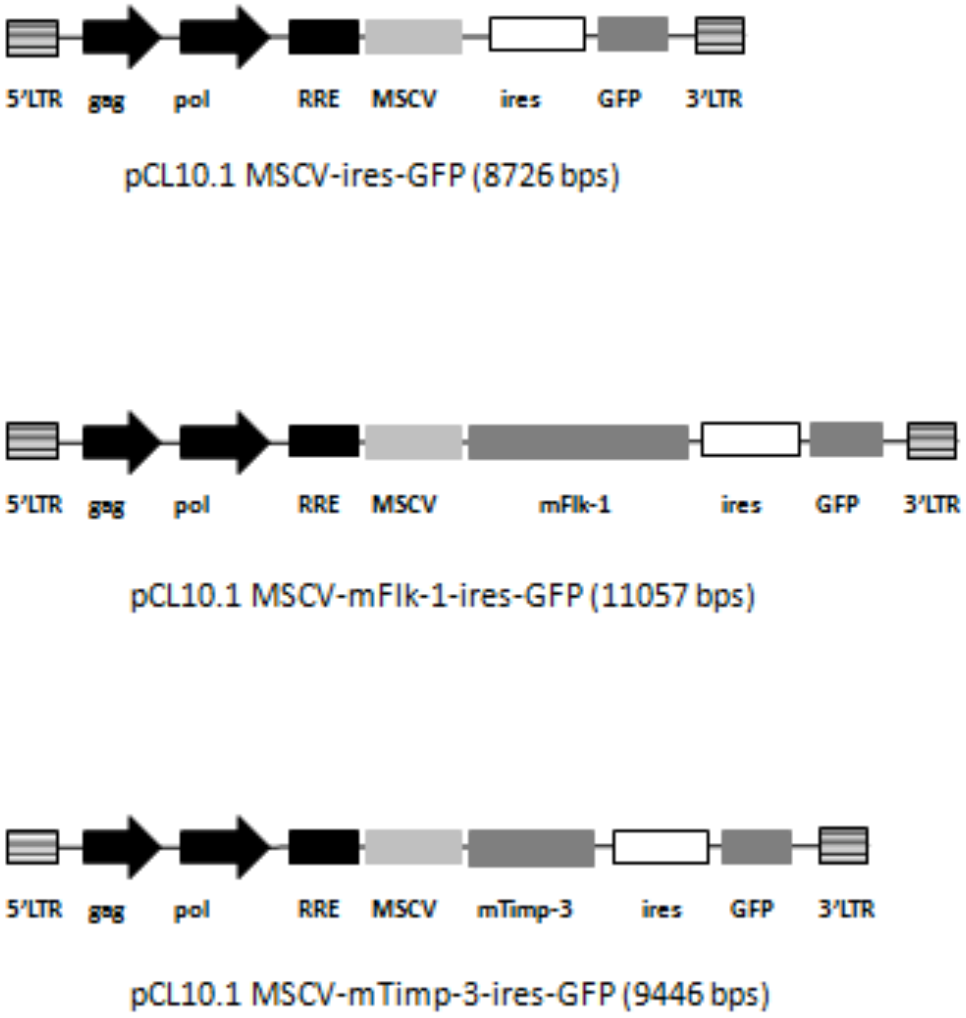


Figure 4.1 Schematic diagram of MSCV-ires-GFP, MSCV-mFlk-1-ires-GFP and MSCV-mTimp-3-ires-GFP lentiviral vectors. LTR, long terminal repeat; gag, gene encoding structural proteins; pol, gene encoding viral enzymes; RRE, REV responsive element; MSCV, murine stem cell virus promoter, ires, internal ribosome entry site

4.2.2 Lentiviral vector production, purification and quantitation

Lentiviral supernatants were made by a 4 plasmid transient transfection method using 293 T cells (for details see chapter 2). Following transfection viral supernatant was harvested at 36, 48 or 72 hours, centrifuged at 1500rpm (400G) for 5 minutes to remove any cell debris and passed through a 0.2micron cell filter. Purified virus was concentrated by ultracentrifugation and its biological activity quantified by transducing HeLa cells with polybrene and determining the proportion of GFP positive cells by FACS analysis. The optimum time to harvest virus following transfection was determined to be 48hrs (Figure 4.2). The mean biological titre of the TIMP-3-GFP and Flk-1-GFP lentiviral vector supernatants was approximately $1x10^6$ transducible units (TU)/ml pre-concentration and $0.5\text{-}1x10^8$ TU/ml following ultracentrifugation. Higher titres were achieved with the control IR-GFP vector of $5x10^6$ TU/ml pre-concentration and $1\text{-}3x10^8$ TU/ml post concentration.

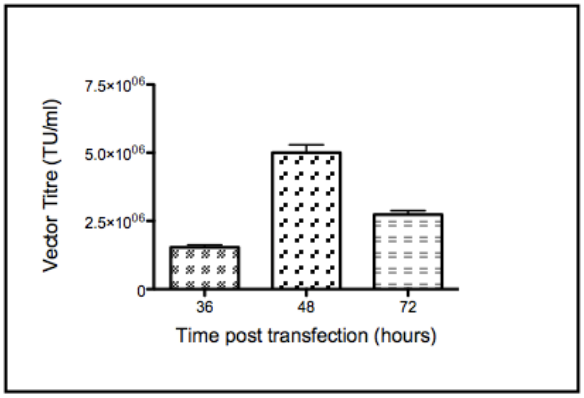


Figure 4.2 Lentiviral vector titer quantitation by Hela cell transduction pre-concentration (histograms show mean values, error bars represent S.E.M)

4.2.3 Functional assessment of lentiviral vectors

Having shown that the lentiviral preps were biologically active by transducing HeLa cells we proceeded to determine whether transduced cells were able to express the vector encoded protein. Analysis of supernatants from transduced cells confirmed Flk-1 and Timp-3 expression by Western Blot (Figures 4.3, 4.4). It was also important to ensure that the lentiviral vector genome did not undergo rearrangement during the transduction process. To prove this, Southern blots were performed on transduced HeLa cells using a radiolabelled probe against the GFP fragment. We were able to demonstrate the correct

size band corresponding to the GFP fragment in Timp-3, Flk-1 and IR transduced cells with no extra bands thus confirming the absence of any genomic rearrangement (Figure 4.5).

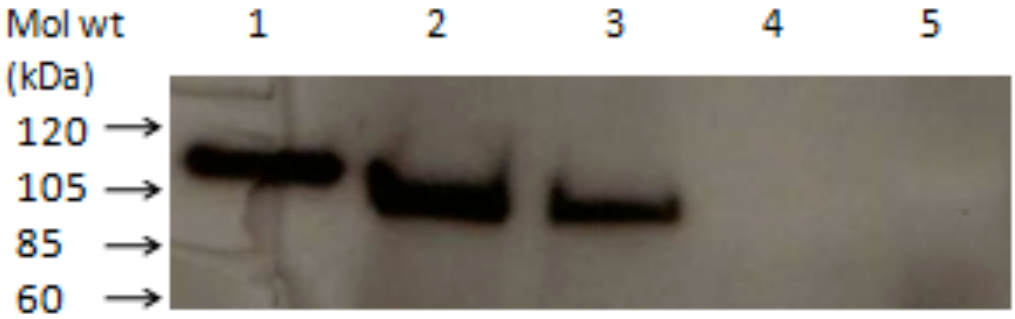


Figure 4.3 Western blot showing tsFlk-1 expression in the supernatant of 293T cells transduced with MSCV-Flk-1-ires-GFP lentiviral vector. Lane 1 recombinant murine Flk-1 protein, lanes 2 and 3 sFlk-1 transduced 293T cell supernatants, lanes 4 and 5 untransduced 293 T cell supernatants. 10μg of total protein loaded per well of a 10% gel and run under reducing conditions.

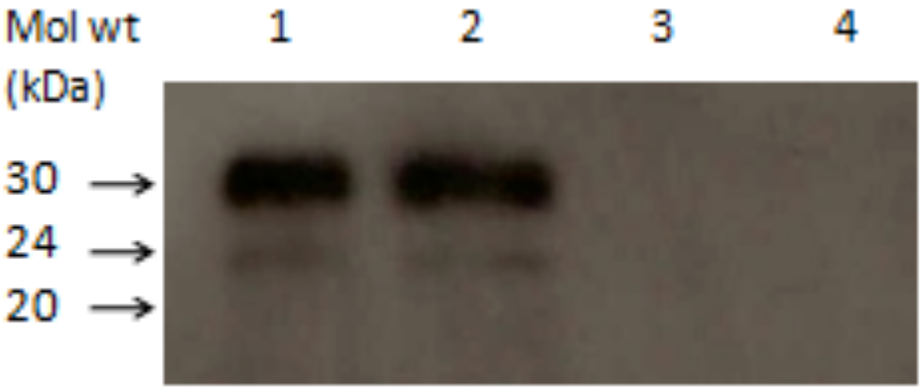


Figure 4.4 Western blot showing Timp-3 expression in the supernatant of KMSBM myeloma cells tranduced with MSCV-Timp-3-ires-GFP lentiviral vector. Lanes 1 and 2 KMSBM-Timp3 cell supernatant showing glycosylated (30kDa) and unglycosylated (24kDa) Timp-3, lanes 3 and 4 untransduced KMSBM cell supernatant. 50μl of supernatant harvested from $1x10^6$ tumour cells was loaded per well of a 15% gel and run under reducing conditions.

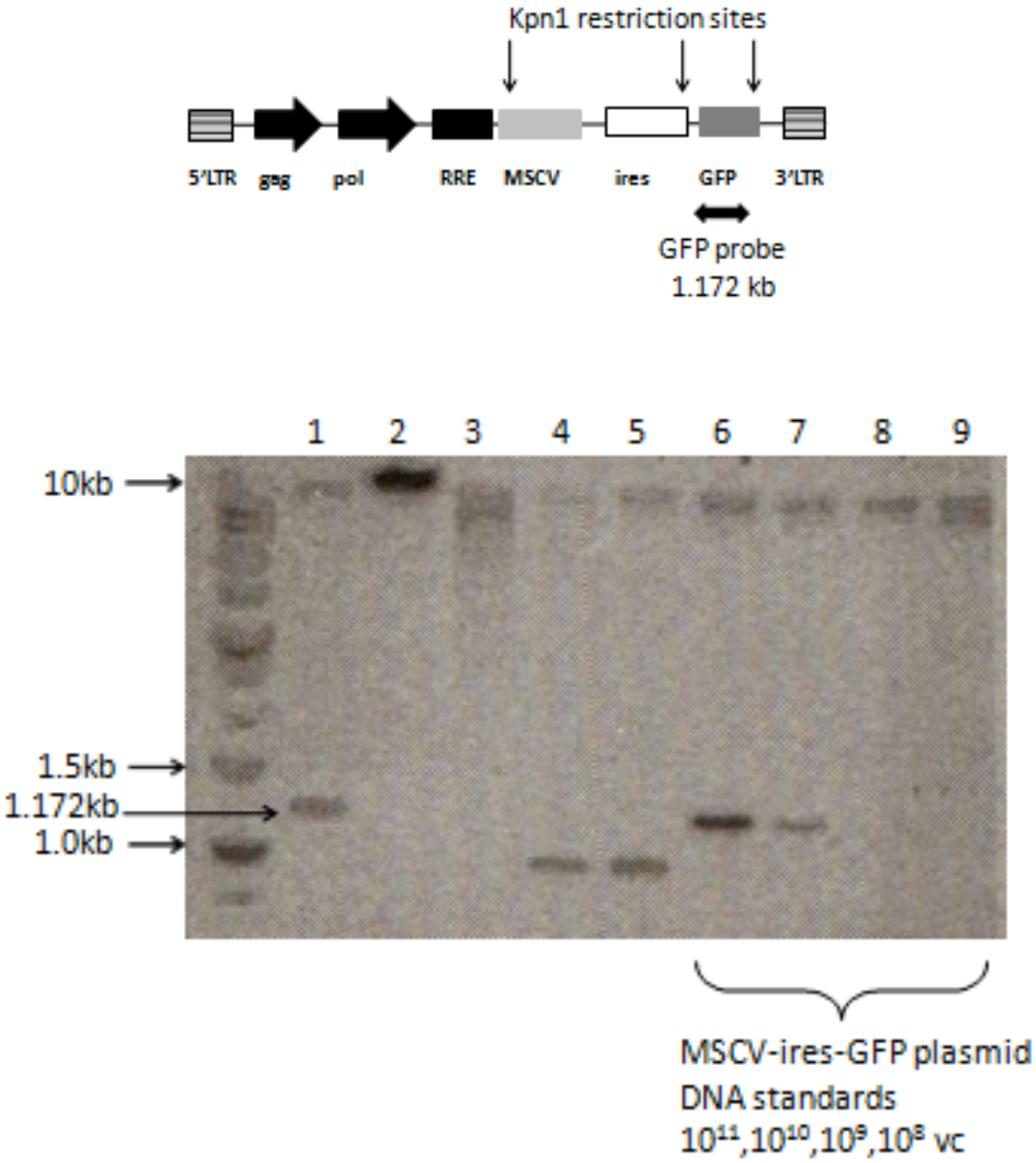


Figure 4.5 Southern blot analysis showing correct integration pattern of the lentiviral vector pCL 10.1 MSCV-ires-GFP. Genomic DNA (70µg) from lentivirally transduced Hela cells was subjected to Kpn1 restriction enzyme digest (lane 1) or was run undigested (lane 2). The predicted 1.172 kb band was seen after kpn1 digest. Controls included kpn1 digest of genomic DNA from previously characterised oncoretroviral MSCV-ires-GFP vector transduced cells (lanes 4 and 5 showing predicted 921bp fragment) and pCL10.1 MSCV-ires-GFP plasmid DNA standards (lanes 6-9) containing 10^{11}-10^{8} vector copies. The probe was directed against a GFP containing fragment as shown in the schematic diagram above.

The transduction efficiency of these vectors on a variety of haematological cell lines was investigated. All tumour cell lines were efficiently transduced with >90% GFP positivity achieved with MOIs of 10 (Table 5.1). Some tumour cells like the ARH77 plasma cell leukaemia cell line needed to be multiply transduced to achieve >90% GFP positivity with the mean intensity of fluorescence also being lower, presumably reflecting lower vector copy numbers. We were also able to show long term persistence of the transgene through multiple passage of transduced cells.

4.3 Recombinant adeno associated viral vectors

4.3.1 Cloning of rAAV vectors encoding sFlk-1, hIFNβ and hFIX

Recombinant AAV plasmid pAV CAGG-hFIX was originally cloned by Dr Nathwani as described and consists of the CMV-IE enhancer, β-actin promoter, chicken β-actin/rabbit β-globin composite intron (CAGG), 1.6kb human FIX cDNA and a rabbit β-globin polyadenylation signal flanked by the AAV inverted terminal repeats (Nathwani et al., 2001). Recombinant pAV CAGG mFlk-1 plasmid was created by excising the mFlk-1 cDNA from AdExFlk.6His plasmid and ligating it with the pCAGGS plasmid (Davidoff et al., 2002) (Figure 4.6).

4.3.2 rAAV 2/2, 5/5 and 2/8 vector production, purification and quantitation

A three plasmid transient transfection method was used to make rAAV vector (see chapter 2). Virus was packaged with either AAV2, AAV5 or AAV8 plasmids, purified by heparin affinity or ion exchange chromatography and concentrated by centrifugation. The purity of the prep was assessed by examination of the clarity of capsid proteins on Coomassie Blue stained SDS-PAGE gels. Figure 4.7 shows a representative gel of rAAV vector post purification, demonstrating the three distinct capsid proteins with minimal other contaminating bands. Viral preps were quantified by DNA slot blots and the average titre was determined to be $1\text{-}3\times10^{11}$ vector genomes/ml (vg/ml) (Figure 4.8).

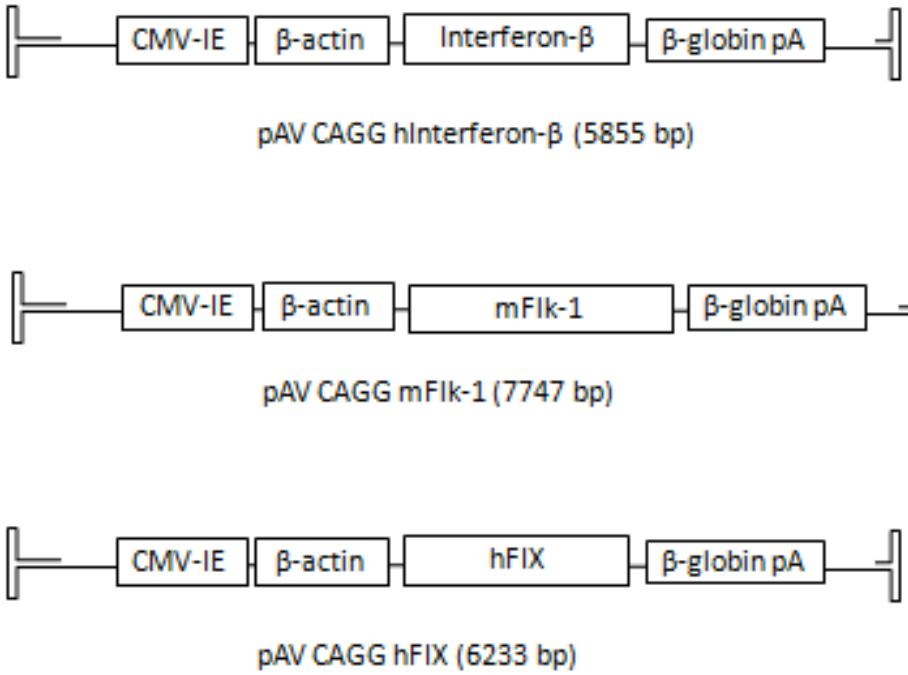


Figure 4.6 Schematic diagram of pAV hIFNβ, pAV mFlk-1 and control pAV hFIX vectors. All constructs had a cytomegalovirus immediate early enhancer (CMV-IE), a β-actin promoter, a chicken β-actin/rabbit β-globin composite intron and a rabbit β-globin polyadenylation signal mediating the expression of either human interferon-β, soluble murine Flk-1 or human factor IX. The expression cassette was flanked by either AAV-2, AAV-5 or AAV-8 inverted terminal repeats (ITRs).

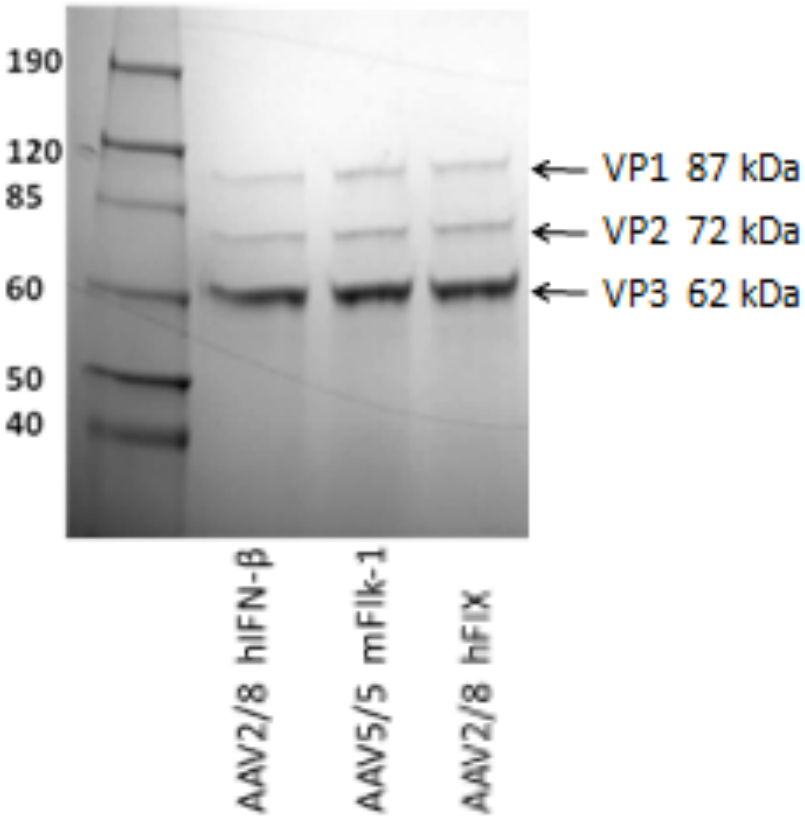


Figure 4.7 Coomassie Blue stained gel of three different rAAV vector stocks post purification showing capsid proteins of molecular weights 87, 72 and 62kDa. 50µl of purifed virus was electrophoresed on a 12% SDS-PAGE gel under denaturing conditions and stained with coomassie blue.

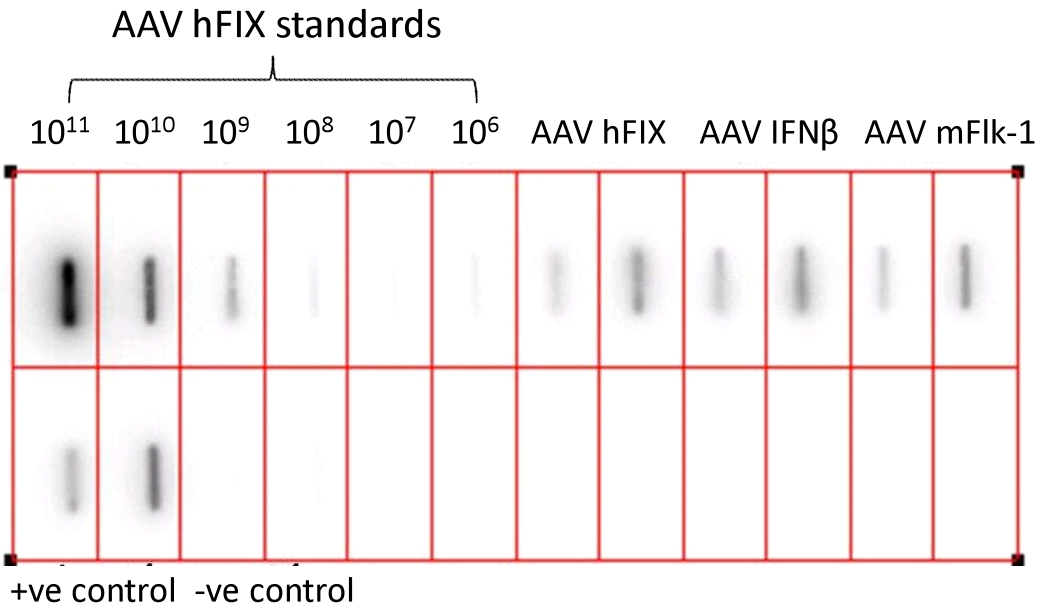
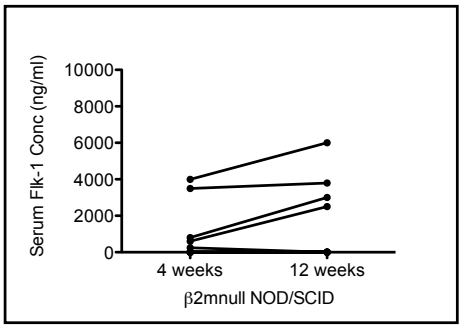
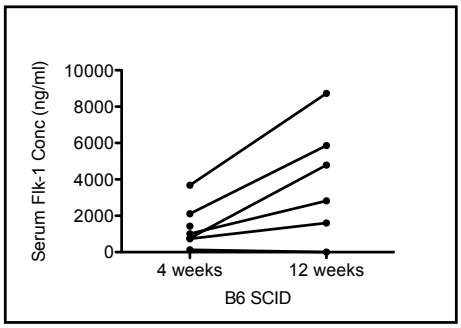


Figure 4.8 Quantitation of rAAV8 hFIX, IFN-β and rAAV5 mFLK-1 vectors by slot blot analysis. 50µl of three different purified AAV vector stocks were denatured and transferred onto a nylon membrane at two different dilutions followed by hybridisation with a radiolabelled probe against the chicken β-actin/ rabbit β-globin (CAGG) sequence. rAAV2/8 hFIX vector standards ranging from 10^{11} to 10^{7} genomes/ml as well as positive (previously titred rAAV2/8 hFIX vector) and negative controls (AAV8 hFIX plasmid) were also transferred onto the membrane. The amount of AAV vector DNA was quantified by measuring the amount of radioactivity on the membrane for each sample and comparing it with the standard curve generated from the rAAV2/8 hFIX standards.

4.3.3 Transduction efficiency of rAAV vectors in vivo

Previous studies had suggested that AAV5 and AAV8 were superior to AAV2 for in vivo gene transfer (Davidoff et al., 2005a). We therefore used these serotypes to evaluate transgene expression in the two different mouse strains used in our animal models namely β2m^{null}NOD/SCID and B6-SCID mice. In our standard experiments each animal was given a vector dose of 1×10^{11} vgs by tail vein injection. Mice were bled at two weekly intervals and serum levels of the protein of interest measured by ELISA. Serum protein levels were detectable at four weeks following AAV5 injection and as early as two weeks following AAV8 challenge, eventually reaching a plateau at 8 weeks (data not shown). Peak levels were higher with AAV8 than AAV5. Furthermore serum Flk-1 levels were consistently higher in B6-SCID mice than in the β2m^{null}NOD/SCID strain (Figure 4.9).





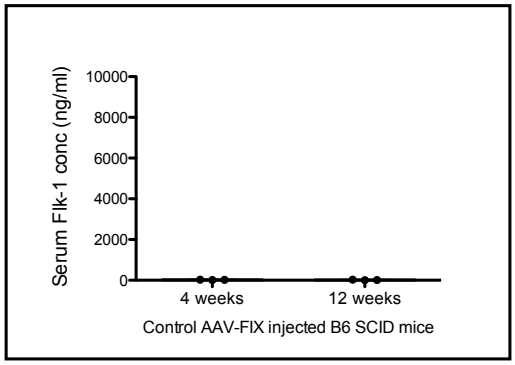


Figure 4.9 Comparision of serum Flk-1 levels between two different strains of immunodeficient mice following tail vein injection of 2.5 x 10^{11} AAV8 vector copies – results from β2m^{null}NOD/SCID mice (n=5), B6 SCID mice (n=6) and control AAV-FIX injected B6SCID mice (n=3) shown

The biodistribution of AAV was also evaluated following in vivo transduction by PCR of murine tissue. As shown in Figure 4.10 the liver appears to be the predominant site of transgene expression although vector particles can be detected to some extent in most other organs by QPCR (data not shown).

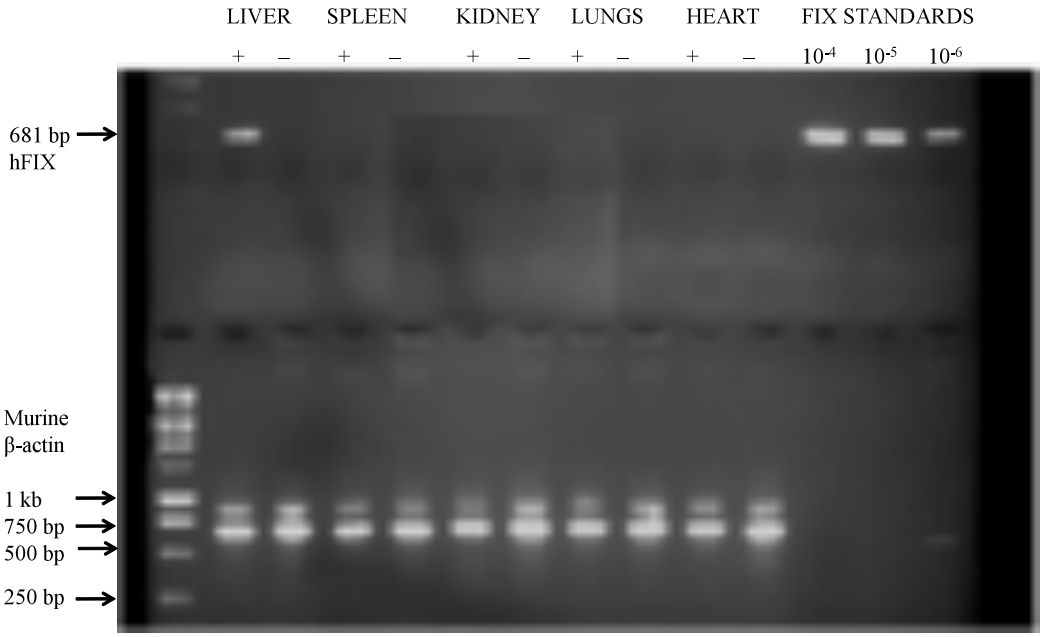


Figure 4.10 Factor IX transgene expression within liver of mice transduced systemically with 2.5x10^{11} vector copies of rAAV8-hFIX (transduced mice + , control mice —). 1μg of genomic DNA extracted from murine organs (12 weeks post transduction) was subjected to PCR using primers that amplified a 681bp region of hFIX cDNA. Standards consisting of serial dilutions of AAV-hFIX vector DNA in negative genomic DNA were used to quantitate proviral copy number. Simultaneous PCR of the housekeeping murine β-actin gene (604 bp region + larger splice variant) was performed to ensure equivalent DNA amounts for each sample. 10μl of the 50μl PCR reaction was run on a 1.5% agarose gel as shown above.

4.4 Discussion

We have optimised the conditions for production of purified high titre lentiviral vectors. The ability of our lentiviral constructs to transduce a wide variety of haematological cell lines with high efficiency was demonstrated. Long term transgene expression as a result of lentiviral vector integration into the host cell genome was clearly shown.

With respect to rAAV vectors, we have again been able to develop a methodology to produce reasonably pure, high titre AAV5 and AAV8 vectors. The transduction efficiency of AAV5 and AAV8 vectors on haematopoietic cell lines in vitro is not much higher that with AAV2 vector but in vivo we have shown high and long term transgene expression following systemic administration of both these vectors, with AAV8 serotype

giving the best results. The liver specificity of the AAV constructs was confirmed by the relatively high level of transcripts seen in murine liver tissue compared with other organs.

At the outset of my work the lentiviral and AAV vector constructs had already been cloned but the functional evaluation of these constructs was done entirely by myself. Similarly optimisation of the lentiviral vector production protocol was performed by myself. The AAV vector purification protocol was developed in conjunction with my colleague Jenny McIntosh.

Having optimised the conditions for production of our lentiviral and rAAV vectors and assessed their transduction efficiency in vitro and in vivo, we were then able to proceed towards evaluating the effect of viral vector mediated antiangiogenic gene transfer on haematological tumour progression in xenograft models.

CHAPTER 5 : EFFECTS OF TISSUE INHIBITOR OF METALLOPROTEINASE-3 EXPRESSION IN XENOGRAFT MODELS OF MULTIPLE MYELOMA AND ACUTE MYELOID LEUKAEMIA

5.1 Introduction

Matrix metalloproteinases (MMPs) play an important role in promoting angiogenesis and tumour growth. They are mainly produced by stromal cells as well as many tumour cells and are responsible for proteolytic degradation of the extracellular matrix, a prerequisite for angiogenesis as well as tumour metastases. They also have direct effects on tumour and endothelial cells with both pro and antiapoptotic properties. A positive correlation has been shown between MMP expression and the invasive and metastatic potential of many solid tumours including colon, lung, breast, ovarian and prostate carcinoma. A number of synthetic MMP inhibitors have been developed which have shown impressive antitumour effects in preclinical solid tumour models. Their results in clinical trials however have been largely disappointing.

MMP activity is regulated by the Tissue inhibitors of metalloproteinases (TIMPs 1-4). There has been increasing interest in the use of these endogenous MMP inhibitors, in particular TIMP-3, for their antiangiogenic and potentially antitumour effects. TIMP-3 is distinct from the other TIMPs in having a high binding affinity for the extracellular matrix as well as having a greater specificity for the adamalysin metalloproteinases (ADAMs) in addition to inhibiting most MMPs (Leco et al., 1994; Murphy et al., 2003). It is proapoptotic and uniquely has the capacity to block angiogenesis directly by preventing VEGF binding to its signaling receptor VEGFR-2 (Qi et al., 2003). In vitro studies have shown that over expression of TIMP-3 in melanoma, cervical carcinoma and sarcoma cell lines inhibited their ability to invade through Matrigel (Ahonen et al., 1998; Baker et al., 1999). Furthermore TIMP-3 over expression was shown to inhibit tumour formation in murine xenograft models of melanoma, squamous cell carcinoma and breast carcinoma (Ahonen et al., 2002; Anand-Apte et al., 1996). However the therapeutic effects of TIMP-3 in haematological malignancies had not been evaluated previously. We therefore investigated the effects of TIMP-3 over expression in xenograft models of myeloma and acute myeloid leukaemia and the results of these experiments are described in this chapter.

5.2 Results

5.2.1 Exogenous TIMP-3 does not affect proliferation of KMSBM, ARH-77 and HL-60 cell lines in vitro

Recombinant murine TIMP-3 (rmTIMP-3) at varying concentrations (0-1 mg/ml) was added to myeloma and acute myeloid leukaemia cell lines in vitro, in the exponential

phase of growth. No obvious change in cell proliferation was observed at 24, 48 and 72 hours by MTS assay even at the highest concentration of TIMP-3 (Figures 5.1A, 5.2A).

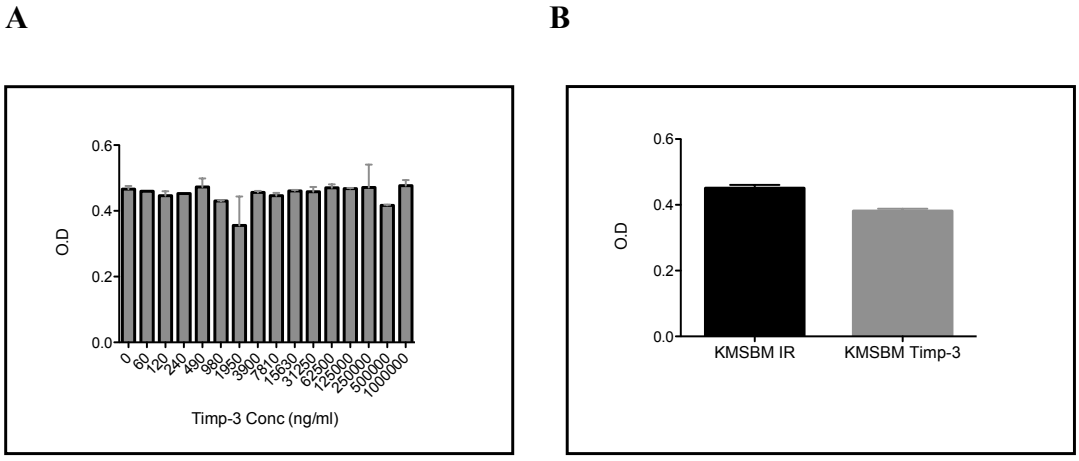


Figure 5.1 MTS assays showing the effects of exogenous (A) and endogenous (B) Timp-3 on the proliferation of KMSBM cells at 72 hours. O.D. measurements were done in triplicates (mean and S.E.M values shown on graph) with the assay repeated twice. No correlation was seen between recombinant Timp-3 concentration and KMSBM proliferation (Pearson rank correlation p=0.577) and no statistically significant difference was seen with endogenous Timp-3 expression (Mann Whitney U test p=0.072)

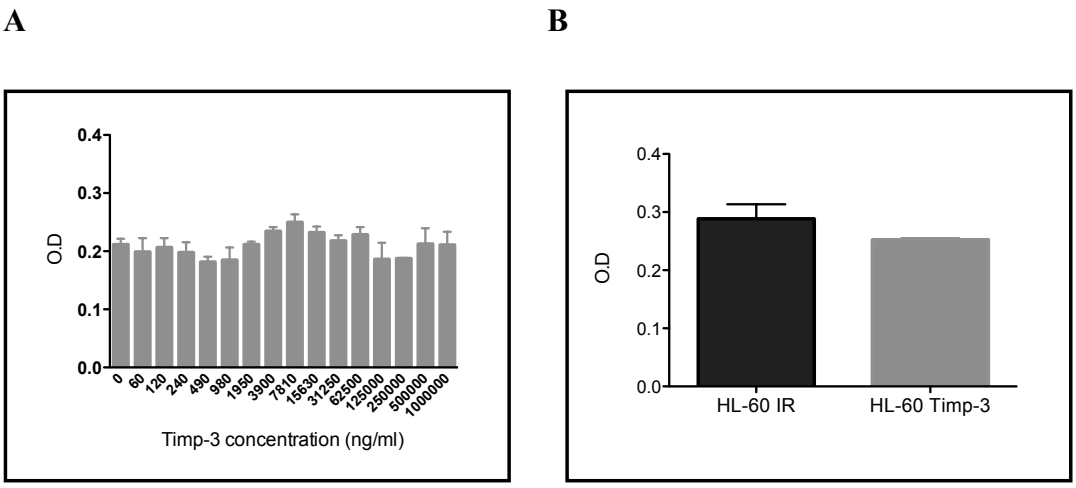


Figure 5.2 MTS assays showing the effect of exogenous (A) and endogenous (B) Timp-3 on the proliferation of HL-60 cells at 72 hours. O.D. measurements were done in triplicates (mean and S.E.M values shown on graph) with the assay repeated twice. No

correlation was seen between recombinant Timp-3 concentration and HL-60 proliferation (Pearson rank correlation p=0.876) and no statistically significant difference was seen with endogenous Timp-3 expression (Mann Whitney U test p=0.070)

C

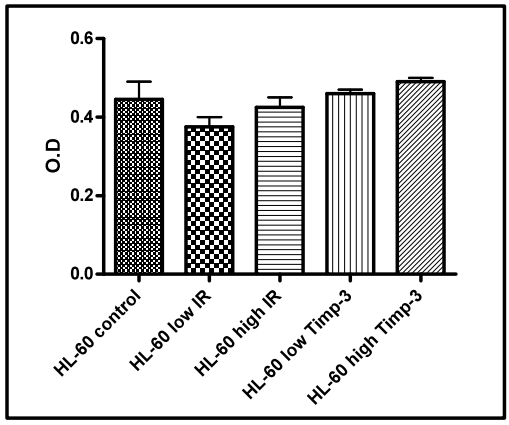


Figure 5.2 C. MTS assay showing the effect of different levels of Timp-3 endogenous expression on the proliferation of HL-60 cells at 72 hours. Mean of triplicate O.D measurements shown with S.E.M error bars. No significant difference between groups by one way ANOVA test (p=0.195)

5.2.2 Endogenous TIMP-3 expression does not affect proliferation of KMSBM, ARH-77 and HL-60 cell lines in vitro

We next sought to determine whether TIMP-3 could have a direct effect on tumour cell proliferation when expressed endogenously. Tumour cells were transduced with bicistronic lentiviral vectors encoding either murine TIMP-3 and GFP or GFP alone. Transduction was performed on consecutive days in serum free conditions in the presence of polybrene, using an MOI of 10 (see Methods). Variable levels of transduction were seen depending on the type of tumour cell. Transduction efficiency was highest with KMSBM cells and lowest with ARH-77 (Table 5.1). All transduced cell lines were sorted for GFP by flow cytometry to give a 100% GFP+ve population. Furthermore transduced HL-60 cells were also sorted into low and high GFP expressing cells. No difference in cell proliferation was seen between TIMP-3 and control GFP transduced cells by MTS assay at 48 or 72 hours (Figures 5.1B, 5.2B). Furthermore there was no difference in proliferation between the low and high TIMP-3-GFP expressing HL-60 cells (Figure 5.2C).

Table 5.1 Transduction efficiency of Flk-GFP, Timp-3-GFP and control IR-GFP lentiviral vectors on myeloma and leukaemia cell lines.

Vector	HL-60 (%GFP)	KMSBM (%GFP)	ARH77 (%GFP)
IR-GFP	90.9	98	11.3
Flk-GFP	63.1	96	9.1
Timp-3-GFP	82	72	8.7

5.2.3 Effect of TIMP-3 expression in xenograft murine models of myeloma and leukaemia

5.2.3.1 Effect of TIMP-3 expression in the KMSBM myeloma model

The effect of TIMP-3 expression in vivo against myeloma cells was then investigated using the KMSBM myeloma model. In this model tumour infiltration within the bone marrow of $\beta 2m^{null}$NOD/SCID mice occurs within six weeks of tumour injection leading to hind limb paralysis (see Chapter 3 for details). Cohorts of $\beta 2m^{null}$NOD/SCID mice were injected intravenously with 1×10^7 TIMP-3-GFP (n=5) or control GFP (n=5) transduced KMSBM cells. In both cohorts tumour cells were 100% GFP positive prior to injection. Six weeks after tumour injection, levels of tumour infiltration in both cohorts were assessed by FACS analysis of bone marrow single cell suspensions and confirmed by immunohistochemistry.

FACS analysis showed a significant difference in tumour infiltration between the two cohorts as measured by the proportion of GFP expressing cells in bone marrow ($p=0.015$ Mann Whitney U test). The mean proportion of tumour cells in the TIMP-3-GFP cohort was 4.68% (±1.04) compared with 48.43% (±10.53) in the control GFP cohort (Figure 5.3).

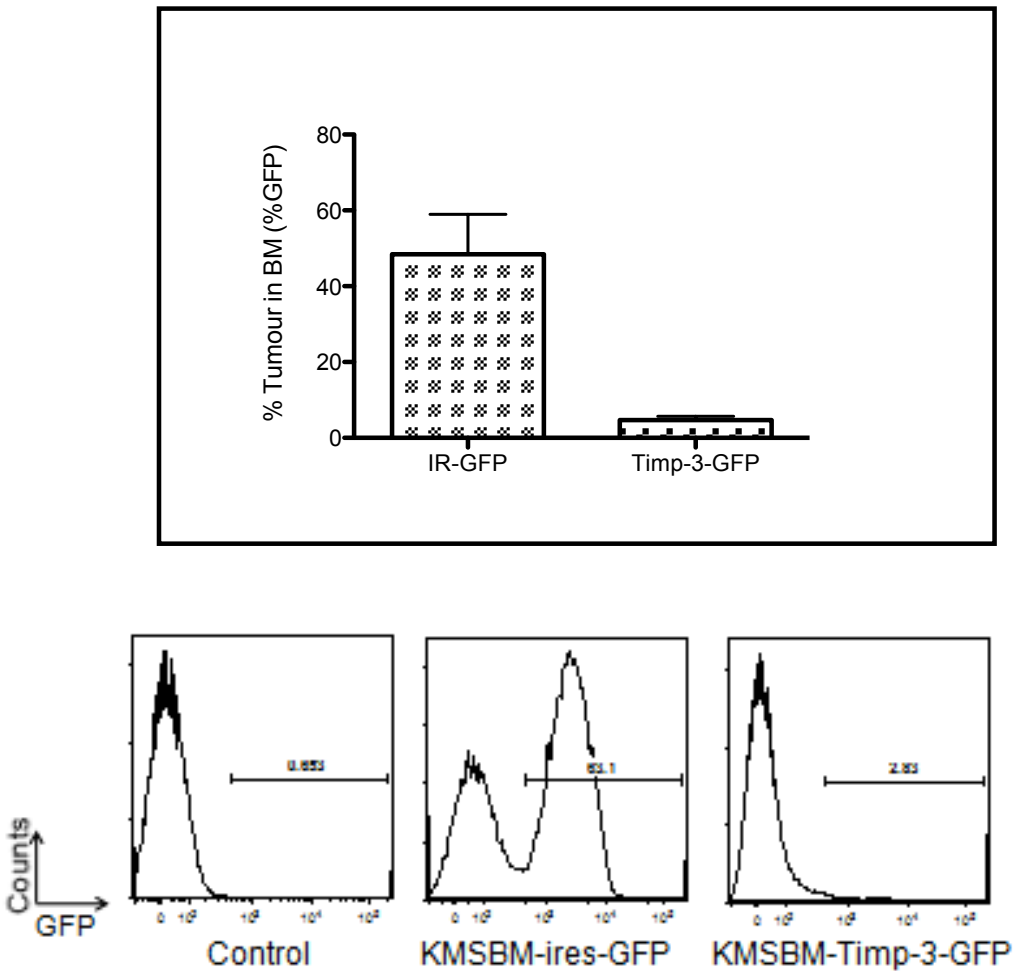


Figure 5.3 FACS analysis demonstrating significantly reduced tumour burden in bone marrow of mice injected with Timp-3 transduced KMSBM cells compared with control IR-GFP KMSBM injected mice. Top histogram shows mean tumour infiltration (error bars show S.E.M) within bone marrow in a cohort of 4 KMSBM-ires-GFP mice compared with that in a cohort of 5 KMSBM-Timp-3-GFP mice. Representative FACS plots shown in lower panel.

Immunohistochemistry using anti-GFP and anti-VS38c antibodies also confirmed the inhibitory effect of TIMP-3 expression on KMSBM tumour infiltration in vivo. Within the IR-GFP cohort 4 out of 5 mice showed high levels of bone marrow tumour infiltration (1 early death occurred from infection) compared with the Timp-3-GFP cohort where 3 out of 5 had no evidence of disease and the remaining two had only minimal disease (Figure 5.4).

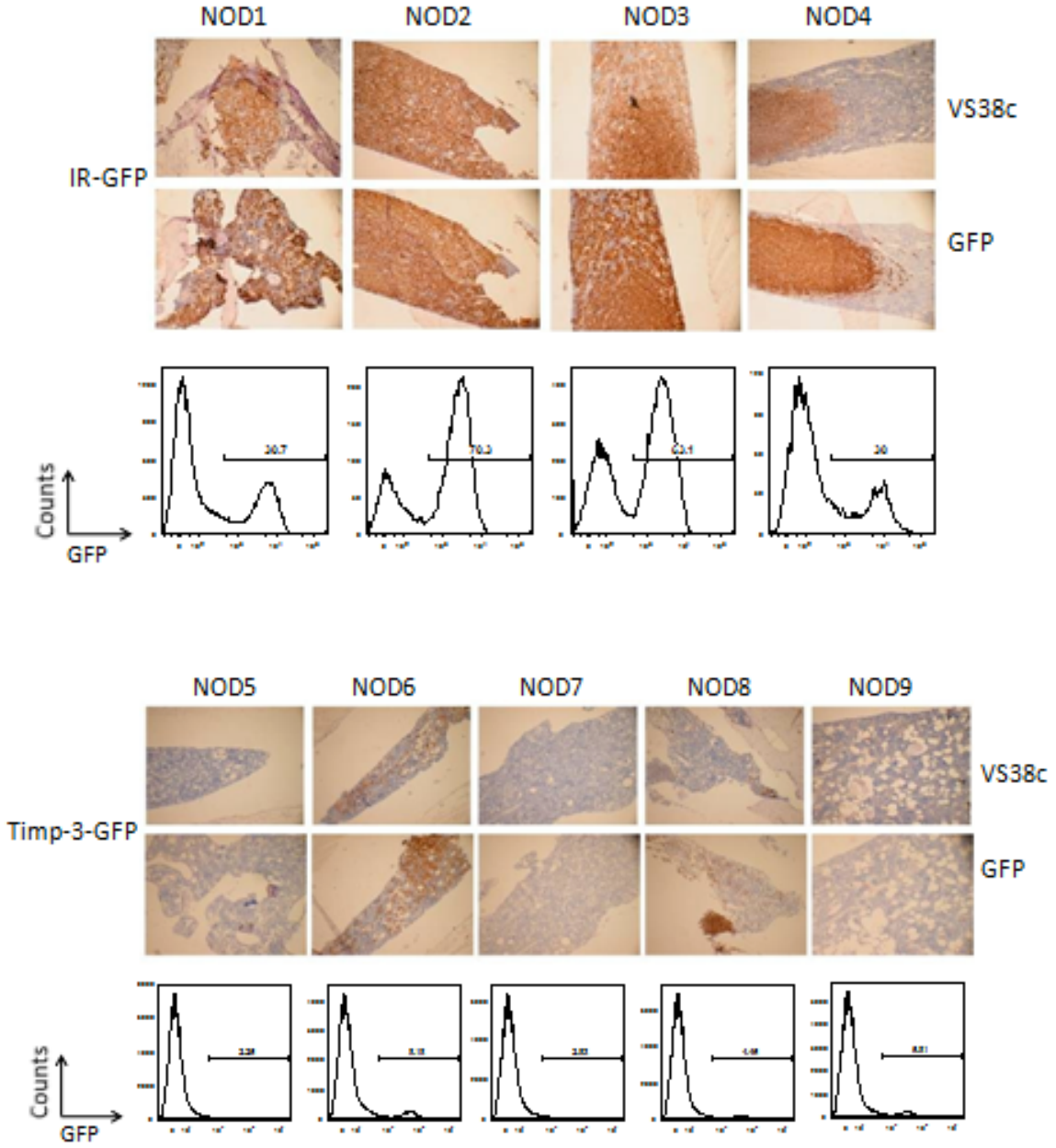


Figure 5.4 Immunohistochemistry and FACS analysis showing tumour infiltration of murine bone marrow from two cohorts of mice infused with either KMSBM-ires-GFP or KMSBM-Timp-3-GFP Bone marrow from mice injected with IR-GFP (NOD1-4) or Timp-3-GFP transduced KMSBM cells (NOD5-9) was harvested at 6 weeks post tumour injection and paraffin embedded sections stained with either anti-VS38c or anti-GFP antibodies. Single cell bone marrow suspensions were also analysed for GFP positivity by FACS analysis as shown above.

5.2.3.2 Effect of TIMP-3 expression in the ARH77 myeloma model

To determine whether the inhibitory effect of TIMP-3 expression in vivo would be seen with other myeloma cell lines, the above experiment was repeated with transduced ARH77 cells. As before tumour cells used for these in vivo experiments were 100% GFP positive. TIMP-3-GFP or control GFP transduced ARH77 cells were then injected into $\beta 2m^{null}$ NOD/SCID mice (n=4 for each cohort). Tumour infiltration in bone marrow was assessed at four weeks following tumour inoculation. Tumour load was found to be low in all mice but there was no significant difference seen between the TIMP-3-GFP and control GFP cohorts (Figure 5.5).

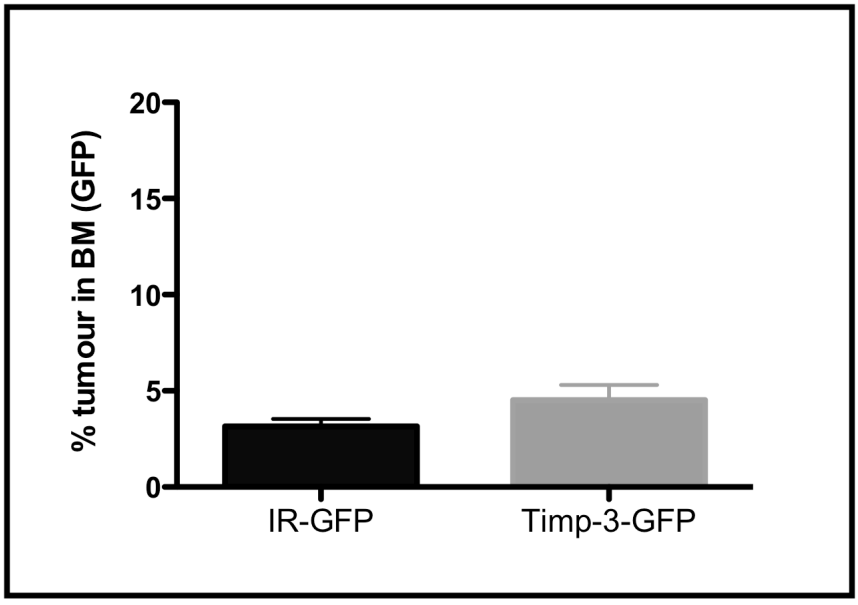


Figure 5.5 FACS analysis showing no significant difference in tumour burden between ARH77-Timp-3 and control IR-GFP tumour injected mice Histograms show mean GFP percent values from a single experiment involving 4 mice in each group, error bars represent S.E.M. Mann Whitney U test shows no significant difference between IR transduced or Timp-3 transduced ARH-77 groups (p= 0.342)

5.2.3.3 Effect of TIMP-3 expression in the HL-60 leukaemia model

The effect of TIMP-3 expression on HL-60 cell proliferation in vivo was also evaluated in a similar way. TIMP-3-GFP or control GFP transduced HL-60 cells were injected intravenously into cohorts of $\beta 2m^{null}$ NOD/SCID mice (n=6 for each cohort). Four weeks later mice were culled and tumour load assessed in each of the mice by GFP FACS

analysis and immunohistochemistry. Variable levels of tumour infiltration were seen in bone marrow and spleen but with no significant difference between the TIMP-3-GFP and control GFP cohort (Figure 5.6).

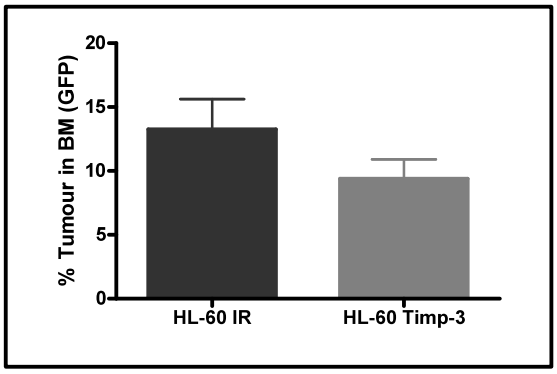


Figure 5.6 Comparision of tumour infiltration by GFP FACS analysis between cohorts of mice given control transduced or Timp-3 transduced HL-60 leukaemic cells. Histograms show mean values from a single experiment involving 5 mice in each group, error bars represent S.E.M. Mann Whitney U test shows no significant difference between IR transduced or Timp-3 transduced HL-60 groups (p= 0.428)

5.3 Discussion

Our work has clearly demonstrated that TIMP-3 does have a profound antitumour effect on KMSBM myeloma cells in vivo when expressed endogenously. The mechanisms underlying the antitumour effect of TIMP-3 in this model include inhibition of extracellular matrix degradation thereby preventing angiogenesis and tumour invasion, and direct blockade of VEGF binding to its receptor thereby directly inhibiting endothelial as well as tumour cell proliferation. The possibility that TIMP-3 expression may have negatively influenced homing of KMSBM tumour cells to the bone marrow cannot be entirely ruled out. No significant antitumour effects were seen with the ARH77 myeloma cell line or the HL60 AML cell line in vivo. There are several possible reasons for this difference. Firstly it might be that TIMP-3 has a greater apoptotic effect against KMSBM cells than ARH77 or HL-60 cells in vivo despite not having any discernible direct effect on any of these tumour cell lines in vitro. Secondly it is possible that angiogenesis has a more important role in KMSBM tumour progression than with ARH77 or HL60. Thirdly the level of TIMP-3 expression may be crucial in determining

the extent of its antiangiogenic and antitumour effects. FACS analysis shows 100% GFP positivity in all three of the transduced cell lines but mean fluorescence was significantly higher in KMSBM cells compared with ARH77 or HL-60 indicating that TIMP-3 expression in KMSBM cells was also likely to be correspondingly higher. Furthermore the expression levels of tumour secreted MMPs may differ between the tumour cell types requiring higher levels of TIMP-3 in some tumours to produce the same degree of antiangiogenesis and tumour inhibition.

CHAPTER 6 : EFFECTS OF VEGF PATHWAY BLOCKADE IN MODELS OF ACUTE MYELOID LEUKAEMIA AND NON-HODGKIN'S LYMPHOMA

6.1 Introduction

VEGF is one of the principal proangiogenic factors involved in regulating tumour associated neovasculature, not only in solid tumours but also in haematological malignancies like AML and myeloma. Current evidence supports the hypothesis that haematological tumour cells, endothelial cells (ECs) and bone marrow stromal cells (BMSCs) closely interact with each other in the bone marrow to facilitate tumour progression, mediated to a large extent by VEGF. VEGF expression has been demonstrated on most AML, myeloma and lymphoma cell lines and primary cells as well as BMSCs (Bellamy et al., 1999; Fiedler et al., 1997). VEGF has been shown to promote endothelial cell proliferation and migration (Rousseau et al., 2000). Studies have also demonstrated that VEGF inhibits apoptosis and stimulates proliferation and migration of leukaemic and myeloma cells through autocrine and paracrine pathways (Dias et al., 2000; Podar et al., 2001). The biological effects of VEGF are mediated predominantly through the VEGFR-1 and VEGFR-2 receptors which are found on all adult vascular endothelial cells as well as most leukaemic and myeloma cells. Results from VEGFR knockout mouse models (Fong et al., 1995; Shalaby et al., 1995) and experiments involving blockade of VEGF receptors with antibodies (Dias et al., 2000) have strongly suggested that VEGFR-2 (also termed KDR or Flk-1) is the main receptor involved in endothelial and tumour cell proliferation/migration whilst VEGFR-1 (Flt-1) is more responsible for vascular remodelling.

We have investigated the effects of blocking VEGFR-2 in xenograft models of AML and lymphoma by viral vector mediated gene transfer of the truncated, soluble, ligand-binding extracellular domain of the murine Flk-1 receptor. The soluble Flk-1 receptor can potentially inhibit the VEGF pathway firstly by binding either tumour secreted human VEGF or host stromal tissue secreted murine VEGF and secondly through a dominant negative mechanism by forming non functional heterodimers with endogenous VEGF receptors present on both tumour cells and host endothelial cells. We have also evaluated the effects of blocking the VEGF pathway using a humanised anti-VEGF antibody (Bevacizumab, Genetech) in these models. This antibody binds predominantly tumour secreted human VEGF with minimal binding to murine VEGF (Yu et al., 2008).

6.2 Results of VEGF pathway blockade in AML and lymphoma models

6.2.1 Expression of sFLK-1 and hFIX in vivo following AAV viral vector mediated gene transfer in β2m^{null} NOD/SCID mice

In this experiment 2.5×10^{11} vgs of either AAV5/5 sFlk-1 or AAV5/5 hFIX viral vector per animal were injected into the tail veins of two cohorts of β2m^{null} NOD/SCID mice. Subsequent expression of sFlk-1 and hFIX in vivo was evaluated by ELISA at timepoints of 4 and 12 weeks. In the sFlk-1 transduced cohort (n=5) plasma sFlk-1 levels of 490-3700 (median 720) ng/ml were detected at 4 weeks, rising to 2500-5800 (median 3400) ng/ml at 12 weeks (Figure 6.1). Similarly plasma hFIX levels of 200-350 (median 300) ng/ml were detected in the control hFIX transduced cohort (n=5).

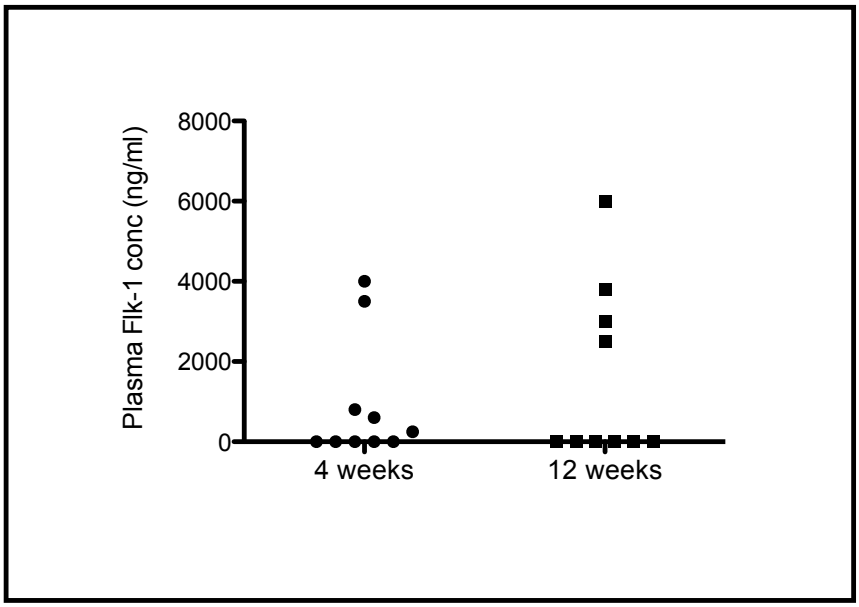


Figure 6.1 Plasma Flk-1 levels in β2m^{null} NOD/SCID mice transduced with AAV5/5 Flk-1 vector .

6.2.2 Effect of sFLK-1 expression in a leukaemia xenograft model

Having established stable systemic transgene expression, 1×10^{7} HL-60 leukaemic cells were injected intravenously into both sFlk-1 and control hFIX transduced mice. At 4 weeks following tumour injection, with several animals now showing signs of being unwell, all mice were culled and tumour load evaluated. There was no significant difference in average spleen weight between the sFlk-1 and control hFIX transduced

cohorts. FACS analysis using anti-human CD45 antibody showed no significant difference in tumour load within bone marrow or spleen between the two cohorts (Figure 6.2). Immunohistochemistry with anti-human CD45 antibody also confirmed that there was a similar degree of leukaemic infiltration in both cohorts of mice (data not shown).

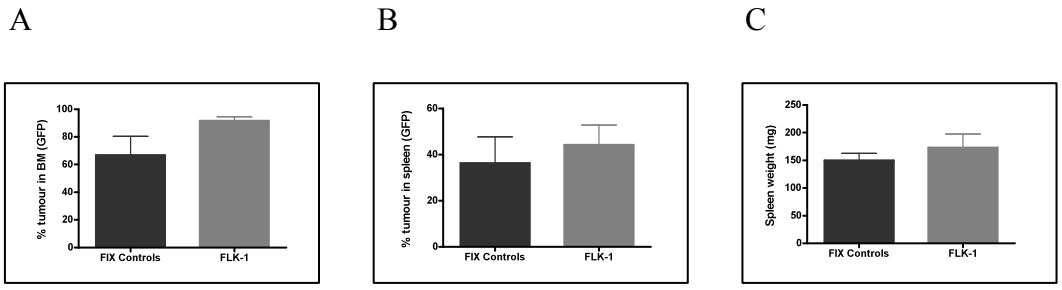


Figure 6.2 FACS data showing no difference in HL-60 tumour infiltration of bone marrow (A) or spleen (B) between FIX and sFLK-1 transduced mice (p=0.142 and p=0.571 respectively, Mann Whitney U test). Plot C shows comparision of spleen weights between the two cohorts (p=0.45 Mann Whitney U test).

6.2.3 Effect of sFLK-1 expression in a lymphoma xenograft model

For this experiment 1×10^{11} vgs of AAV5/5 sFlk-1 or AAV5/5 hFIX vector were injected into cohorts of B6 SCID mice. At 4 weeks following gene transfer, plasma samples were screened for sFlk-1 expression by ELISA and levels of 128-3679 (median 4800) ng/ml were detected in the sFlk-1 transduced cohort (n=7) which increased to 1605-8730 (median 4786) ng/ml by 14 weeks (Figure 6.3A). hFIX expression was confirmed in the control cohort (n=5).

Having established stable transgene expression, 1×10^{7} Raji lymphoma tumour cells were injected intravenously into each animal from both cohorts. Mice were closely monitored and culled at the earliest signs of disease related distress or hind limb paralysis. The presence and extent of tumour infiltration were evaluated by hCD45 immunohistochemistry. Expression of sFlk-1 failed to protect the mice from developing tumour infiltration as demonstrated by the survival curves in Figure 6.3B. There was a trend for sFlk-1 expressing mice to die earlier than controls but the difference was not statistically significant (p value =0.5).

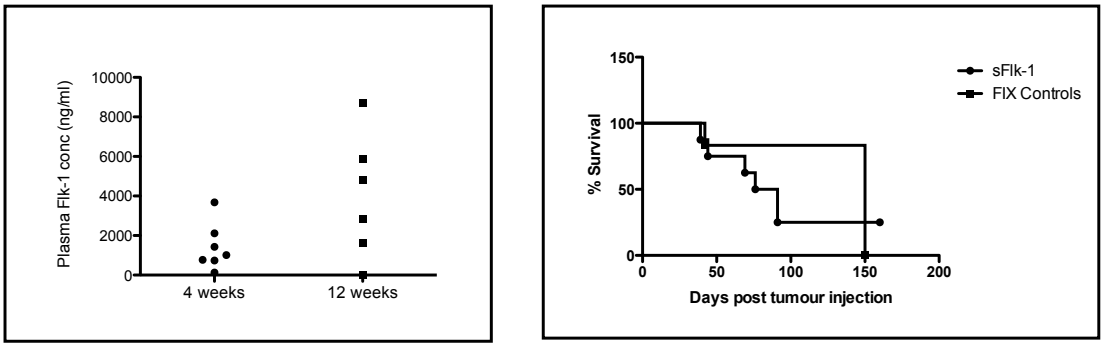


Figure 6.3 A. Serum sFlk-1 levels by ELISA in B6 SCID mice which were injected with 1x10^7 Raji tumour cells 6 weeks after transduction with AAV5-sFlk-1 (left) B. Kaplan-Meir plots showing no improvement in survival in these mice compared to Raji injected hFIX expressing mice (right) p=0.505 Log-rank test.

6.2.4 Effect of VEGF pathway blockade using a recombinant humanised anti-VEGF antibody in a leukamia xenograft model

An alternative method of blocking the VEGF pathway is to use a monoclonal antibody directed against VEGF (Bevacizumab). We evaluated the effect of using a recombinant humanised anti-VEGF antibody in our leukaemia xenograft model. Three cohorts of $\beta 2m^{null}$ NOD/SCID mice were injected intravenously with 1×10^7 HL-60 cells per animal. A week later two of the cohorts were injected with 5×10^{10} viral genomes of either AAV5/5 sFlk-1 (n=6) or AAV5/5 hFIX (n=6) per animal intravenously. Animals from the third cohort (n=6) were each injected with 5mg/kg of anti-VEGF antibody intraperitoneally three times a week. Expression of sFlk-1 in the AAV sFlk-1 transduced cohort was confirmed by ELISA with a mean plasma sFlk-1 level of 3890 (range 1632-4988) ng/ml. Similarly hFIX expression was confirmed in the control cohort. Stable levels of anti-VEGF antibody in plasma was also demonstrated by ELISA in the antibody treated mice.

A B

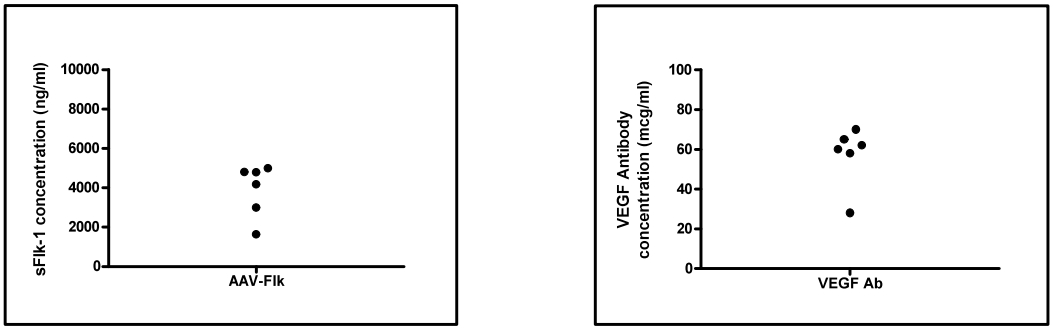


Figure 6.4 Serum` sFlk-1 levels and VEGF Antibody levels measured in AAV-Flk transduced mice (A) and VEGF Antibody treated mice (B) respectively. ELISA done either 4 weeks post AAV injection or 24 hours post antibody injection following 4 weeks of treatment

The effect of sFlk-1 expression and anti-VEGF antibody treatment on serum VEGF levels in vivo was determined. Serum human VEGF levels were measured by ELISA and mean levels from the above three cohorts compared. No significant difference in mean human VEGF levels was seen although levels were generally low throughout (Figure 6.5). Furthermore no difference in serum murine VEGF levels was seen in any of the animals (data not shown).

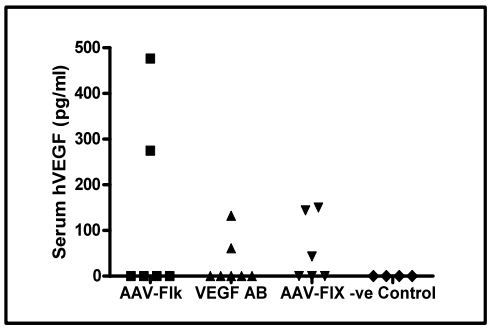


Figure 6.5 Serum human VEGF ELISA showing similarly low levels of human VEGF in HL-60 injected mice regardless of whether they were transduced with AAV-sFlk-1 or AAV-FIX or treated with VEGF antibody (5mg/kg). A cohort of non tumour untransduced control mice was also included.

Analysis of tumour load at the end of four weeks showed no significant difference in the degree of tumour infiltration between the control hFIX and the sFlk-1 transduced cohorts.

However the VEGF antibody treated mice, surprisingly, appeared to have significantly increased tumour infiltration (Figure 6.6).

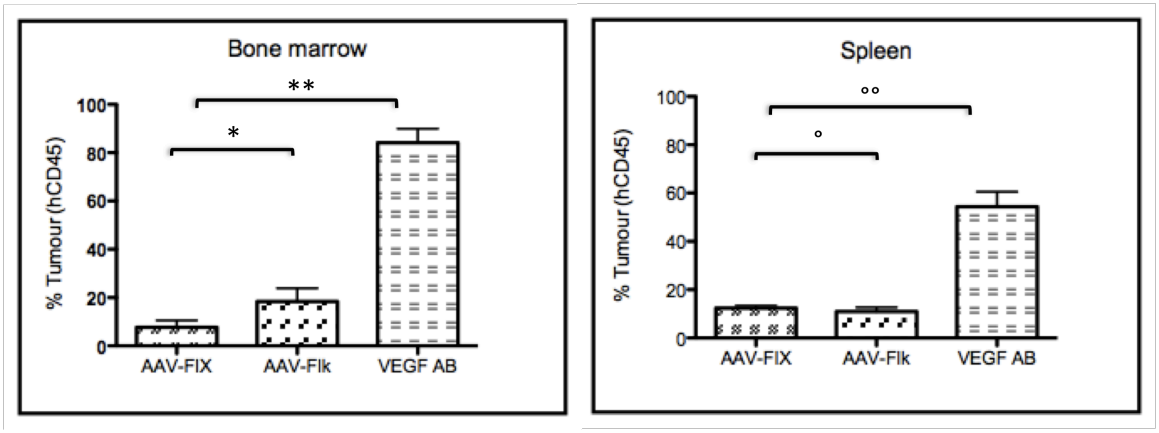


Figure 6.6 FACS analysis results showing tumour burden in bone marrow and spleen of HL-60 injected mice treated with AAV-FIX, AAV-sFlk-1 or VEGF antibody (5mg/kg) Histogram shows mean values from groups of 6 mice with error bars representing S.E.M. *p=0.121 **p=0.0001, °p=0.489 °°p=0.0001 (t test)

In a further experiment the effect of VEGF antibody dose titration on HL-60 cell proliferation in vivo was evaluated. Four cohorts of $\beta 2m^{null}$ NOD/SCID mice were injected intravenously with 1×10^7 HL-60 cells per animal. Three of the cohorts were subsequently treated with anti-VEGF antibody at doses of 0.5, 1 or 5 mg/kg, administered three times a week intraperitoneally (n=4 each cohort). Animals from the control cohort on this occasion were each injected with 12.5 μg/ml of anti RhD antibody using the same regime. Comparision of tumour load at four weeks showed no difference between mice treated with the 5 mg/kg dose of VEGF antibody and the anti RhD treated control mice. However the cohorts treated with the lower doses of antibody did show slightly higher tumour infiltration than the control cohort although this was not statistically significant (Figure 6.7).

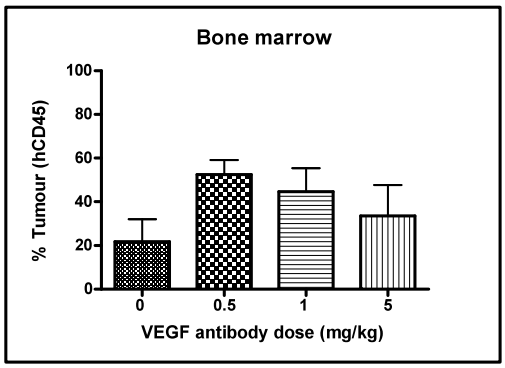


Figure 6.7 FACS analysis results showing effects of VEGF antibody dose on bone marrow tumour burden in HL-60 injected mice Mean values with S.E.M shown from a single experiment with 4 mice per group. No statistically significant correlation was seen p=0.599 (Pearson rank correlation). Control mice (labelled as 0 mg/kg on histogram) were given 5mg/kg of anti-RhD antibody

6.2.4 Effect of anti-VEGF antibody treatment in a lymphoma xenograft model

Two cohorts of irradiated B6 SCID mice were injected with 1×10^7 Raji cells intravenously (n=8 each cohort). A week later one cohort was commenced on VEGF antibody treatment at 5mg/kg intraperitoneally three times a week. The control cohort was treated with anti RhD antibody in a similar way. The time taken for mice to become unwell and develop hind limb paralysis as a result of tumour proliferation was noted. The survival curves in Figure 6.8 show that treatment with VEGF antibody failed to prevent tumour infiltration in this lymphoma model.

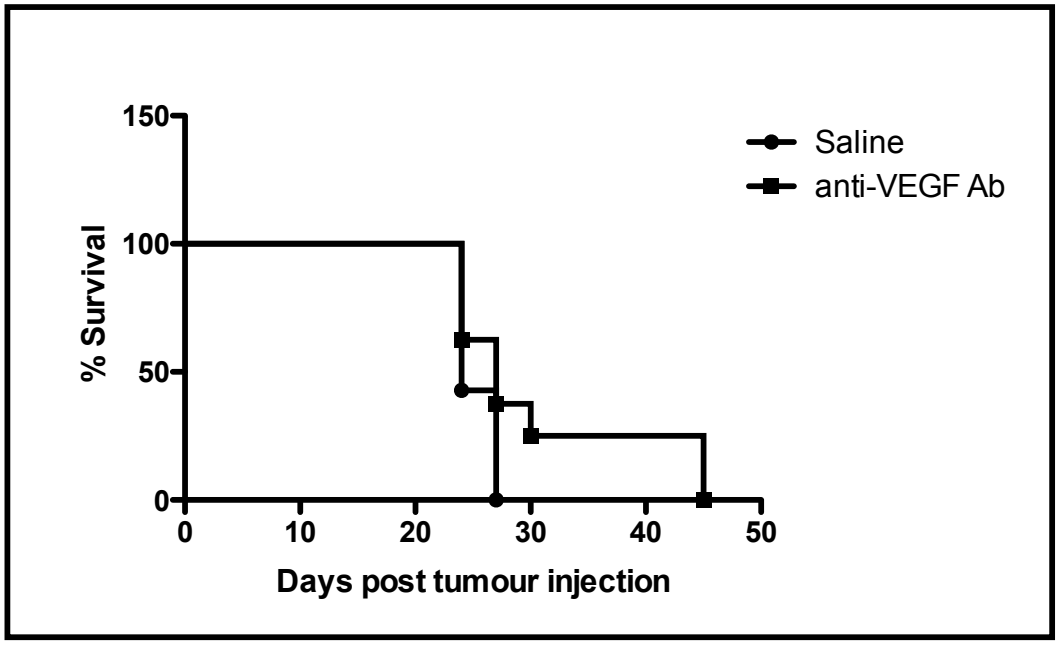


Figure 6.8 Kaplan-Meir plot showing no difference in survival of Raji injected mice between anti-VEGF antibody and saline treated cohorts (p=0.1289 log Rank test, n=8 each cohort)

6.3 Discussion

The results of our work show that blocking the VEGF pathway, either by expressing the soluble Flk-1 receptor or by administering an anti-VEGF antibody, does not result in any significant antitumour effects in our leukaemia or lymphoma xenograft models. One possible explanation for the lack of therapeutic benefit might be that the level of VEGF pathway blockade achieved was suboptimal. However the serum levels of sFLK-1 and anti-VEGF antibody achieved in our in vivo experiments far exceeded the doses previously shown to inhibit angiogenesis in vitro (Davidoff et al., 2002). An alternative explanation is that excessive blockade of the VEGF pathway leads to the development of tumour resistance by a compensatory increase in VEGF levels either from the tumour cell or from host tissue. Such a phenomenon was demonstrated by Davidoff (Davidoff et al., 2005b) in a neuroblastoma murine model where significant antitumour efficacy was seen in mice with low levels of Flk-1 expression in contrast to mice with high Flk-1 levels where tumour progression was much more aggressive. Furthermore tumour resistance in these mice was shown to be mediated by increased VEGF expression from tumour cells and murine tissue. We were unable to demonstrate increased serum levels of murine or human VEGF with Flk-1 expression or anti-VEGF antibody treatment. However serum

VEGF levels can often be quite unreliable and a better measure might be tissue VEGF levels. Further evidence comes from a phase II trial of colorectal carcinoma where the anti-VEGF antibody Bevacizumab was found paradoxically to be less effective at the higher of the two doses used suggesting that optimal inhibition of angiogenesis may depend crucially on the dose of antiangiogenic agent used. Our experiments using 0.5, 1 and 5 mg/kg doses of anti-VEGF antibody were not able to demonstrate therapeutic efficacy against AML cell proliferation in vivo. In fact there appeared to be a greater tumour burden in mice treated with the anti-VEGF antibody compared to controls. Finally it may be that antiangiogenic strategies like VEGF signalling blockade on its own may simply be inadequate to arrest the growth of rapidly proliferating tumour types like AML. A more effective strategy for antiangiogenesis might be to use it in combination with more conventional cytotoxic therapies, in the setting of minimal residual disease.

In summary our work has shown that stably blocking the VEGF pathway either by expressing the soluble Flk-1 receptor or by using an anti-VEGF anyibody does not inhibit tumour progression in our HL-60 AML and Raji lymphoma models. Further work needs to be done looking at different levels of VEGF blockade and by using combination chemotherapeutic agents before abandoning them as potential therapeutic strategies in AML and lymphoma.

CHAPTER 7 : EFFECTS OF INTERFERON-β EXPRESSION IN MURINE MODELS OF ACUTE MYELOID LEUKAEMIA

7.1 Introduction

Interferons (IFNs) are a family of cytokines that have significant antitumour activity. The antitumour properties of the type I IFNs (α/β) are a consequence of their antiproliferative, immunomodulatory and antiangiogenic effects. Despite the impressive antitumour efficacy of IFN-α in xenograft models of solid tumours, the results from clinical trials have been far more disappointing. The main limiting factors in the clinical use of recombinant type I IFNs are their short half-life and the side effects associated with peak levels. IFN-α has been used with variable success in a number of paediatric vascular neoplasms, metastatic melanoma and renal cell carcinoma. In the field of haematological malignancies IFN-α has been shown to be efficacious in hairy cell leukaemia, chronic myeloid leukaemia and multiple myeloma but no obvious therapeutic benefit was seen in AML.

There has been increasing interest recently in the use of IFN-β for the treatment of malignancies with many reports suggesting that IFN-β has more potent antitumour effects than IFN-α. However IFN-β has not previously been evaluated in the treatment of AML. In this chapter we report a detailed evaluation of the antileukaemic effects of human IFN-β in vitro and in a xenograft model of AML by using adeno-associated viral vectors to deliver sustained expression of IFN-β in vivo.

7.2 Interferons-α/β inhibit the proliferation and survival of AML cells in vitro

We compared the activity of recombinant human IFNα-2b and IFNβ-1a against a range of AML cell lines (HL-60, OCI AML5, AML193, KG1α) in vitro using thymidine uptake assays. After 3 days of culture we observed significant but equivalent antiproliferative activity with both hIFN-α and hIFN-β against all AML cell lines. The minimum dose required for both agents was 10 IU/ml with a reduction in proliferation of 50-80% occurring at a dose of approximately 1000 IU/ml. Higher IFN concentrations did not result in any further reduction in AML cell proliferation (Figure 7.1).

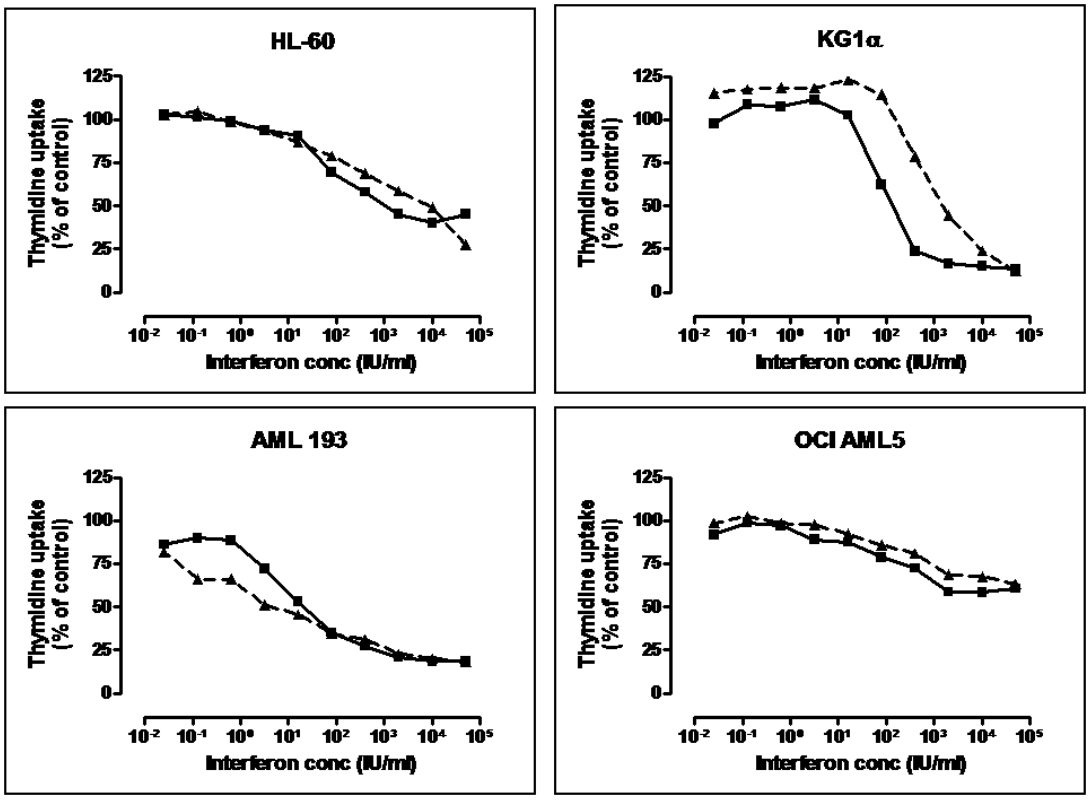


Figure 7.1 Thymidine uptake assays showing the effects of IFNs– α (▲) and β (■) on the proliferation of AML cell lines in vitro. Cells were exposed to recombinant IFNs for 3 days prior to the assay.

We also looked at AML cell survival in response to IFN stimulation using AnnexinV assays and showed upto 50% increase in apoptosis at a dose of 1000 IU/ml after three days of culture (Figure 7.2).

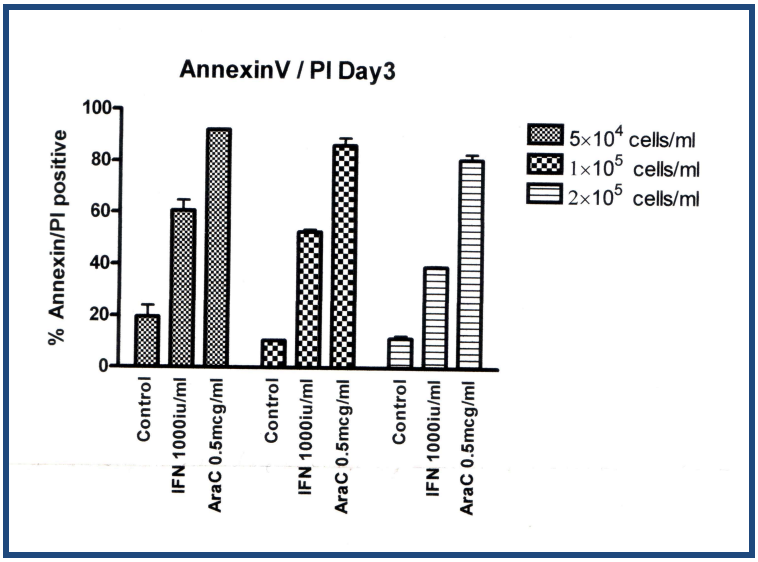


Figure 7.2 Annexin V / Propidium iodide staining of HL-60 cells exposed to 1000IU/ml IFN-β for 72 hours at different cell densities. Cytarabine exposed HL-60 cells were used as positive controls.

7.3 Interferons-α/β affect the cell cycle of AML cells by increasing the fraction in S phase

The effects of type I interferons on the cell cycle were evaluated as described in chapter 2. These experiments showed that IFNs reduced the proportion of AML cells in G1 phase and increased the proportion in S phase (Figure 7.3).

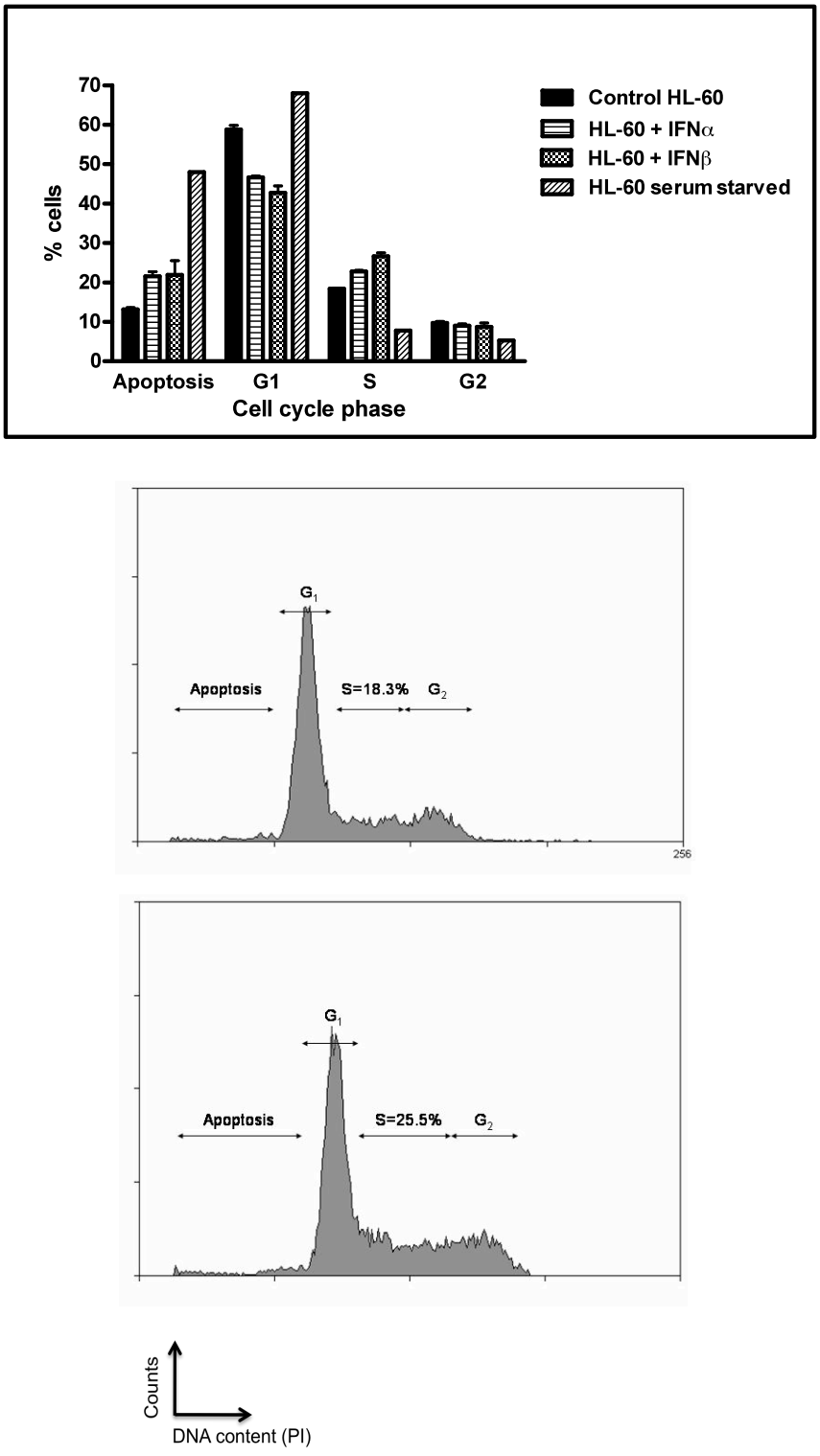


Figure 7.3 Cell cycle analysis of HL-60 cells exposed to 1000 IU/ml of IFNs-α/β for 72 hours Top panel shows mean values with S.E.M of HL-60 cells exposed to different conditions in triplicates and the experiment was done three times. Bottom two panels show the FACS plots of control untreated (middle panel) and IFN-β treated (lower panel) HL-60 cells

7.4 Interferons-α/β induce phosphorylation of the JAK/STAT signalling pathway in AML cells

Previous work has shown that in most cells the biological effects of interferons-α/β are mediated through activation of the Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway following binding of the type I IFN receptor. We have investigated the cell signalling pathways of AML cells in response to IFN stimulation and confirmed the involvement of the JAK/STAT pathway (Figure 7.4).

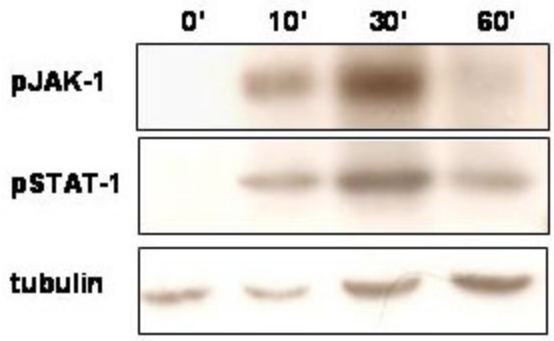


Figure 7.4 Western Blot analysis of HL-60 cells exposed to IFN-β at different time points showing rapid phosphorylation of JAK-1 and STAT-1. Lysates from $2x10^6$ cells exposed to 1000IU/ml IFN-β for 10, 30 or 60 minutes were run on a 10% gel under reducing conditions.

7.5 Effects of interferons-α/β on tumour secretion of angiogenic cytokines

Type I IFNs have been shown to downregulate the production of proangiogenic cytokines by many tumour types. We decided to see if IFNs had a similar effect on AML cells. Following IFN stimulation, HL-60 cells were assessed by PCR for VEGF, MMP-2 and MMP-9 expression. No obvious difference in expression was seen between IFN stimulated and control HL-60 cells (Figure 7.5).

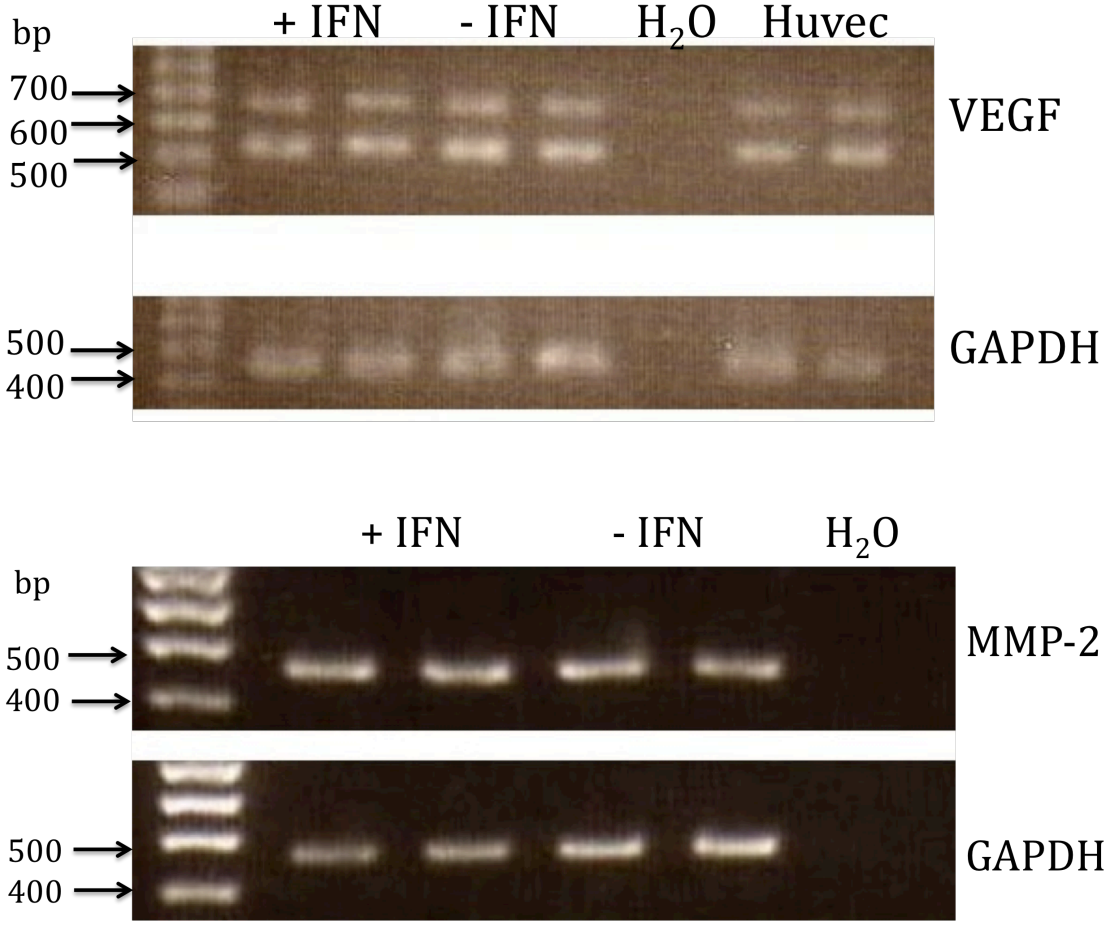


Figure 7.5 VEGF and MMP-2 RT-PCR of HL-60 cells treated with 1000IU/ml of IFN-β for 3 days. 1μg of IFN-β treated HL-60 cell RNA was subjected to PCR in duplicates and 20% of each PCR reaction (10μl) was run on a 1% agarose gel. For the VEGF PCR human endothelial cells were used as a positive control showing the 656 and 584bp isoforms. PCR of the housekeeping gene GAPDH was done simultaneously.

7.6 Recombinant hIFN-β fails to inhibit HL-60 proliferation in a xenograft model of AML but stable expression of interferons-α/β by AAV mediated gene transfer results in significant antileukaemic effects

Having demonstrated the antileukaemic activity of hIFN-α and hIFN-β in vitro, we next assessed their in vivo efficacy in a xenograft model of AML. We chose to focus on hIFN-β because of its reported stronger antiproliferative activity against other tumour types. In our initial experiments recombinant hIFN-β was administered either subcutaneously (2000 IU/mouse thrice weekly) or intravenously (60,000 IU/mouse daily) into cohorts of $\beta 2m^{null}$ NOD/SCID mice, a week after intravenous injection with HL-60 cells with an

additional untreated cohort (n=10) serving as controls. The dose administered weekly was approximately 3-fold or 200-fold higher respectively than the equivalent standard adult human dose of hIFN-β given clinically. Despite administering such high doses, hIFN-β was undetectable in the plasma of animals treated subcutaneously (n=4) at any time point. In contrast, at 2 hours after intravenous injection (n=9) the mean plasma level of hIFN-β was 400 IU/ml (range 200-1000) which declined to undetectable levels by 24hrs, consistent with its short half life. Four weeks after injection of HL-60 cells, heavy but equivalent levels of tumour infiltration were observed in all three cohorts (Table 7.1). These results contrast with the in vitro effects of hIFN-β on AML cells and may be explained either by its rapid clearance or by its poor biological activity in vivo.

Table 7.1. Outcome of recombinant hIFN-β treatment of HL-60 leukemic mice

Treatment cohort	Proportion of animals with significant disease (>30% infiltration)	'Peak' plasma hIFN-β levels (IU/ml)	'Trough' plasma hIFN-β levels (IU/ml)
Controls	10/10	<0.5	<0.5
s.c. hIFN-β	4/4	<0.5	<0.5
i.v. hIFN-β	8/10*	400 ± 100	<0.5

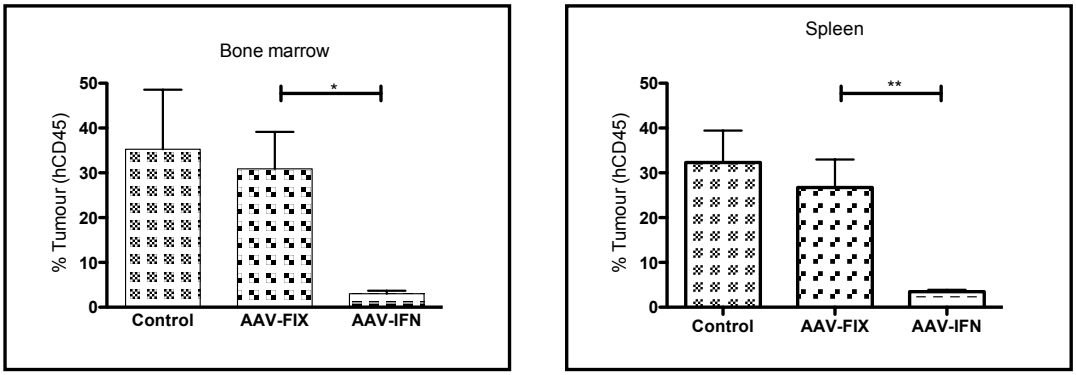
* 2 early deaths from radiation toxicity

Peak levels measured at 2 hours post injection, trough at 24 hours.

To resolve this issue we established stable long-term expression of hIFN-β in vivo following gene transfer with AAV2/8 vectors encoding hIFN-β and subsequently evaluated the effects on AML cell proliferation. β_2m^{null} NOD/SCID mice were injected with HL-60 cells as previously, and a week later AAV2/8 hIFN-β was administered at a dose of $1x10^{11}$ vector particles. Peak plasma hIFN-β levels measuring 20,000 to 100,000 IU/ml were achieved within a week of vector injection and were stably maintained until the mice were culled after four weeks. Six out of eight animals in the hIFN-β transduced cohort had no evidence of leukaemia when analysed by flow cytometry or immunohistochemistry. In contrast, all control mice (n=7) expressing human FIX after AAV mediated gene transfer had widespread evidence of leukaemic infiltration at levels observed in saline treated controls (n=6) (Figure 7.6). These experiments demonstrated

clearly that hIFN-β has a profound antileukaemic effect in vivo providing plasma levels of hIFN-β can be stably maintained.

A



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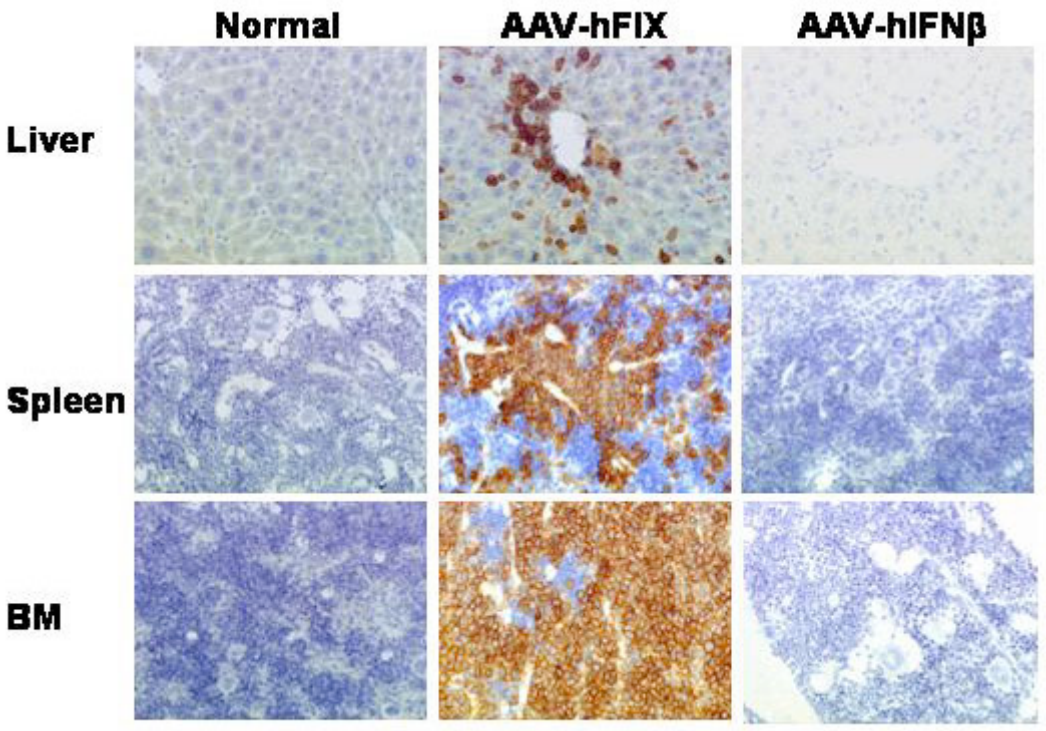

Figure 7.6 (A) HL-60 tumour burden in β2m^{null} NOD/SCID mice following AAV2/8 hIFN-β gene transfer – quantitative results from FACS analysis of bone marrow and spleen single cell suspensions. Control animals included a saline treated cohort and a cohort receiving AAV2/8 hFIX vector. Error bars represent SEM (*p=0.003, **p=0.001) (B) Human CD45 immunohistochemistry confirming the widespread

tissue infiltration of control hFIX transduced mice with HL-60 cells, in marked contrast to hIFN-β transduced animals.

We next attempted to compare the antileukaemic effects of hIFNα and hIFNβ in vivo. AAV2/8 hIFN-α or AAV2/8 hIFN-β vector was administered to cohorts of HL-60 injected mice at a dose of 3×10^{10} vector particles per animal (n=4 each cohort). Despite the equivalent vector dose given, plasma levels of hIFNβ achieved was approximately twofold higher than plasma hIFNα levels (6000±400 and 3000±400 IU/ml respectively) (Figure 7.7). This variation in levels is likely to have arisen from differences in the biological activity of the two different vector stocks, which is not taken into account by the vector dose. Bone marrow tumour load in both hIFNα and hIFNβ cohorts was substantially lower (10.7±5.4% and 0.9±0.5% respectively) than in control FIX transduced animals (76.3±5%) (Figure 7.7). The higher residual tumour load in the hIFN-α cohort compared with hIFN-β is probably a reflection of lower plasma hIFN-α levels although differences in antitumour activity in vivo between hIFN-α and hIFN-β, as observed in many solid tumors, cannot be excluded. Nevertheless we have shown that stable expression of both hIFN-α and β at supraphysiological levels, results in significant antileukemic activity against HL-60 cells in vivo which appears to be mediated (at least in part) through a pro-apoptotic mechanism.

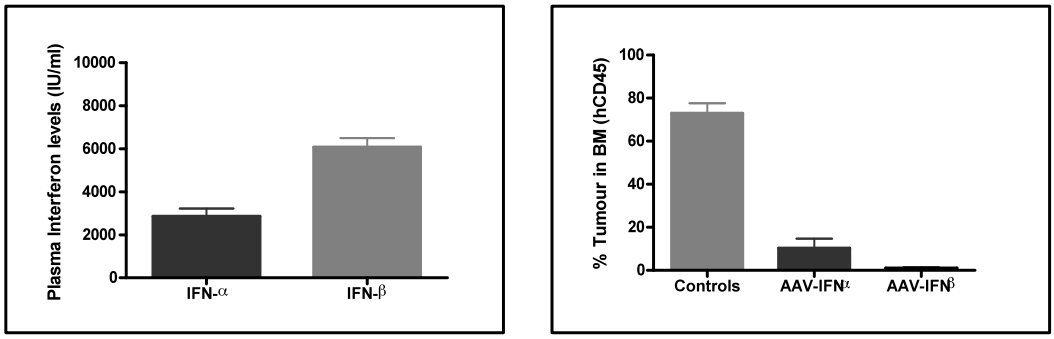

Figure 7.7 Plasma IFN-α/β levels measured by a functional antiviral assay (left). HL-60 tumour burden in bone marrow from mice transduced with either control hFIX, AAV 2/8 hIFN-α or AAV 2/8 hIFN-β - results from FACS analysis.

7.7 Stable expression of interferon-β inhibits primary AML cell proliferation in vivo

We next evaluated the effects of stable hIFN-β expression on the proliferation of primary AML cells in vivo. In separate sets of experiments, $\beta 2m^{null}$ NOD/SCID mice were implanted with $1x10^7$ primary leukemic cells taken from 3 different patients with poor risk AML (high white cell count, Flt3-ITD mutation) followed, two weeks later, by transduction with 1×10^{11} vector particles of AAV2/8 hIFN-β or an equivalent dose of AAV2/8 hFIX. After six weeks mice were analysed and in all 3 experiments there was clear evidence of AML infiltration within the bone marrow of control animals (n=11) whereas none of the hIFN-β transduced mice (n=12) had any evidence of leukaemia (Table 7.2). Plasma levels of hIFN-β in these experiments ranged from 7000 to 90000 IU/ml (median 48000 IU/ml).

Table 7.2 Phenotypic characteristics of AML donors used in these experiments and outcome of engraftment studies following AAV2/8 mediated hIFN-β expression in vivo

	Patient characteristics	Proportion of mice engrafted with AML	
		hFIX transduced	hIFN-β transduced
Patient 1	**AML M4 Flt3/ITD+ve WBC 308**	**3/3**	**0/4**
Patient 2	**AML M1 Flt3/ITD+ve WBC 150**	**5/5**	**0/4**
Patient 3	**AML M1 Flt3/ITD+ve**	**3/4 ***	**0/9**

- **1 death from radiation toxicity**
- **Primary AML engraftment was defined as >1% tumour infiltration of bone marrow by CD45 FACS analysis**

In subsequent experiments three doses of AAV2/8 hIFN-β vector (1×10^{11}, 1×10^{10} or 1×10^9 particles/animal) were administered into cohorts of mice injected two weeks previously with primary AML cells. The degree of AML infiltration within bone marrow was found to correlate directly with mean plasma hIFN-β levels (Figure 7.8) with a 700-fold and 140-fold reduction in tumor load at plasma hIFN-β levels of 55000 ± 12000 and

1500 ± 130 IU/ml respectively. Significantly a 50-fold reduction in primary AML infiltration was seen even when plasma levels were <10 IU/ml (4 ± 0.8 IU/ml). Pharmacokinetic studies in humans have shown a great deal of variability in plasma hIFN-β levels achieved following recombinant hIFN-β treatment but levels under 10 IU/ml have been relatively well tolerated.

A **B**

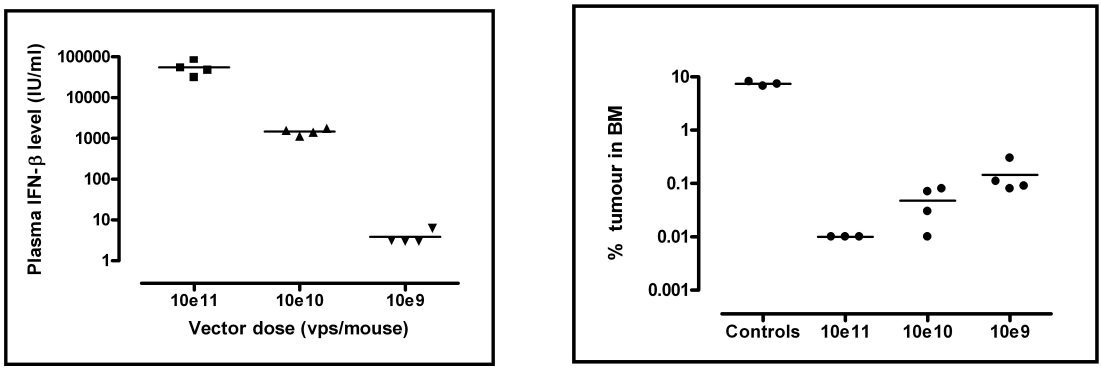

Figure 7.8 (A) Plasma hIFN-β levels in β2m^{null} NOD/SCID mice following AAV2/8 mediated transduction at different vector doses. Measurements made in duplicates on two separate occasions. (B) Effects of plasma hIFN-β levels on primary AML proliferation in vivo - quantitative results from FACS analysis of bone marrow (p<0.01 at all vector doses compared with control)

7.8 Antileukaemic effects of interferon-β in vivo – mechanisms of action

The mechanisms of action underlying the antitumour activity of IFN-β include their direct antiproliferative and apoptotic effects on tumour cells as well as their immunomodulatory and antiangiogenic properties. The use of our immunodeficient β2m^{null}NOD/SCID model enabled us to selectively assess the anti-proliferative and proapoptotic effects of hIFN-β on AML cells in vivo in the absence of its immunomodulatory and direct anti-angiogenic properties as hIFN-β does not cross-react with its cognate murine receptor. We performed TUNEL staining on leukaemic tumours extracted from mice transduced with AAV2/8 hIFNβ and showed increased apoptosis of HL-60 cells compared with tumours from control mice (Figure 7.8) in keeping with our in vitro observations. Attempts at assessing in vivo proliferation with the BrDu assay were not successful (data not shown). Our model did not allow us to examine the direct antiangiogenic effects of hIFNβ but its

potential indirect effects on murine angiogenesis, mediated by modulation of tumour secreted proangiogenic cytokines, were explored by staining murine vasculature. No significant differences, however, were seen in microvessel density between tumours from hIFNβ and FIX transduced animals (data not shown).

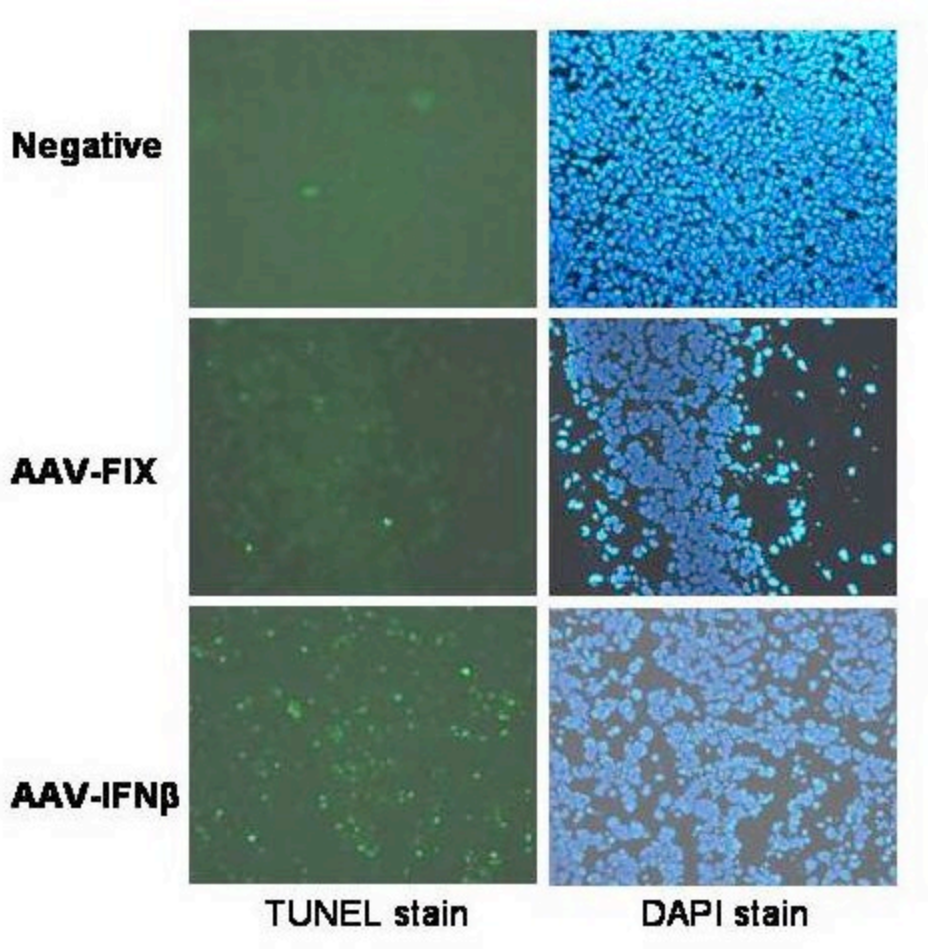

Figure 7.9 TUNEL staining of subcutaneous HL-60 tumour implants following systemic transduction with AAV2/8 hFIX or AAV2/8 hIFN-β. The negative tumour control was stained without prior treatment with TdT enzyme. All slides were counterstained with DAPI

7.9 Discussion

The work presented in this chapter describes the effects of type I interferons (α and β) on AML cell proliferation and survival in vitro and in a xenograft model of AML. We have demonstrated that both IFN-α and IFN-β have profound but equivalent antiproliferative effects against a range of AML cell lines in vitro. This contrasts with the results from

previous studies where IFN-β has consistently been shown to be superior to IFN-α in its antitumour activity against a range of tumour cell lines. We have also shown that type I IFNs have a direct pro-apoptotic effect on AML cells and have proven that the biological effects of type I IFNs on AML cells are mediated, atleast partly, by the JAK/STAT signalling pathway.

Our xenograft model of AML enabled us to study the effects of interferon-β on the proliferation of AML cells in vivo. The use of recombinant human IFN-β failed to demonstrate antileukaemic efficacy in vivo when administered either subcutaneously or intravenously, even when peak plasma levels of 200-1000 IU/ml were achieved with the intravenous route. The most likely explanation for this lack of therapeutic benefit is the short half-life of IFN-β. Pharmacokinetic studies of IFN-β in humans have shown a half-life of approximately five hours with either intravenous or subcutaneous administration (Salmon et al., 1996). It is therefore possible that even frequent parenteral administration of IFN-β may be insufficient to keep the interferon signalling pathway activated for long enough to result in an antileukaemic effect. The short half-life of recombinant IFN-β is consequently a significant limiting factor in clinical trials. Frequent or continuous administration protocols have to be used and the side effects associated with peak levels can often be debilitating. The only published randomized clinical trial of a type I Interferon in AML is that of the UK MRC AML11 trial (Goldstone et al., 2001) where low dose IFN-α (3MIU 3 times a week) was used as maintenance treatment in older patients with AML. No reduction in relapse rate or improvement in survival was shown. Of note was the poor compliance of patients, with only a third of patients able to tolerate the side effects of IFN-α and complete the full 12 months of treatment. An alternative to using recombinant protein is a gene therapy approach where systemic administration of a viral vector would result in transduction and stable low level expression of IFN-β in vivo. The use of AAV8 vectors ensured stable expression of IFN in vivo for the lifetime of the animals thereby circumventing the problem of the short half-life of IFNs. Our experiments clearly show that AAV mediated IFN-α and β expression significantly inhibits HL-60 proliferation and survival in vivo. We were unable to directly compare the antileukaemic effects of IFN-α and β in vivo because of difficulty in ensuring equivalent plasma levels of IFN between the two cohorts. We have also shown for the very first time conclusive evidence of the antitumour effects of IFN-β against primary AML cells in a xenograft model, even when samples from patients with poor prognostic features were used. In these experiments IFN-β gene therapy was initiated a full two weeks after

primary AML cell inoculation showing the effectiveness of IFN-β treatment despite tumour growth being fully established. Furthermore IFN-β was shown to have significant activity against primary AML cells in vivo at plasma levels less than 10 IU/ml, levels which have been shown to be tolerated well in humans.

We have shown that the antitumour activity of IFN-β against leukaemic cells results primarily from its direct antiproliferative and pro-apoptotic properties. However our data also seem to indicate that the antileukaemic activity of IFN-β in vivo is substantially greater than that seen in vitro, thus raising the possibility of other contributory mechanisms. The well known immunomodulatory properties of type I IFNs are unlikely to have played a role in our xenograft model in view of the immunodeficient nature of the β_2m^{null} NOD/SCID mouse strain used. However type I IFNs are known to have antiangiogenic properties which could potentially play a role. Our use of human IFN-β in a murine system rules out a direct antiangiogenic effect because of the species specific nature of interferons. However an indirect antiangiogenic effect arising from downregulation of tumour secreted proangiogenic cytokines in response to IFN stimulation cannot be ruled out. Downregulation of tumour secreted proangiogenic factors bFGF, VEGF, MMP2 and MMP-9 in response to hIFN-β treatment has been demonstrated for a number of solid tumours but we have failed to show a similar effect with HL-60 leukaemic cells. Furthermore we have not shown any obvious difference in MVD in IFN-β treated animals. Another mechanism of action of hIFN-β is the normalisation of the disorganised vasculature of tumours mediated through angiopoietin-1 upregulation, demonstrated in a glioma and neuroblastoma xenograft model following hIFN-β gene therapy (Dickson et al., 2007). We have however not been able to demonstrate such a phenomenon in our hIFN-β treated leukaemia mice.

In summary, our study demonstrates that type I interferons α and β have significant activity against AML in the setting of a xenograft model. Detailed analysis with hIFN-β showed that sustained expression even at low but potentially clinically tolerable levels, achieved here by gene transfer, resulted in significant inhibition of leukemic cell proliferation in vivo. These results suggest that alternative delivery strategies such as the use of continuous intravenous or subcutaneous administration protocols or the longer acting pegylated form of IFN-β or a gene therapy approach may prove more effective in treating patients with AML.

CHAPTER 8 : CONCLUSIONS

8.1 Conclusions

The aim of this thesis was to investigate the therapeutic effects of stable antiangiogenic expression in murine models of Acute Myeloid Leukaemia, Multiple Myeloma and non-Hodgkin's Lymphoma. The hypothesis was that angiogenesis played a crucial role in the pathogenesis of haematalogical malignancies such as AML, MM and NHL through the provision of oxygen and nutrients to the proliferating tumour cells as well as by releasing a number of growth factors and cytokines and therefore blocking key angiogenic pathways would result in inhibition of tumour growth.

It was essential to first develop appropriate murine models of the haematological malignancies we were targeting as antiangiogenic strategies are best evaluated in vivo where tumour associated neovasculature and the extracellular matrix are thought to play an important role in facilitating tumour progression. Prior to our work only few transgenic models existed for AML and lymphoma and none for myeloma. The xenograft models that had been developed were often orthotopic models that bore no resemblance to the human disease and the systemic models that were in use were often limited by unreliable disease phenotype. Commonly used immunodeficient mice strains for tumour engraftment included the NOD/SCID and B6 SCID strains. However as we have shown residual NK cell activity (seen in both these strains) can result in tumour rejection necessitating the use of irradiation or NK cell depleting antibodies. We have developed a range of xenograft models of AML, B cell NHL and MM that closely mimic the human phenotype. The use of the $\beta 2m^{null}$ NOD/SCID strain which lacks B, T and NK cells enabled us to reliably engraft a range of haematological human tumour cell lines in vivo by tail vein injection of tumour cells without the need for additional irradiation. We have thus developed models for AML using the HL-60 cell line and importantly primary AML cells, MM using the ARH-77 and KMSBM cell lines and NHL using both Raji and U937 cell lines. Important features of these models include the systemic nature of the disease, the predilection of the disease for infiltrating bone marrow as well as for causing bone lytic disease in the KMSBM myeloma model and the increased neovasculature seen at tumour sites. Furthermore the slower growth kinetics of the tumour in the primary AML and the KMSBM models makes them potentially more amenable to antiangiogenic strategies.

As we have highlighted there are numerous naturally occurring pro and antiangiogenic molecules found naturally that usually exist in equilibrium. However under the influence of tumours this equilibrium is altered and the angiogenic switch is activated leading to

increased neovasulature which facilitates tumour progression. Based on the evidence at the time we decided to target two key angiogenic pathways – the vascular endothelial growth factor pathway and the matrix metalloproteinase pathway and to investigate the effects of blocking these pathways on tumour progression in AML, MM and NHL models. We also evaluated the effects of expressing human interferon-β, a cytokine with multiple mechanisms of action incuding antiangiogenesis, on AML cells in vivo. Experience from several clinical trials using antiangiogenic molecules and interferons had led to the conclusion that the short half-life of these agents potentially limited their efficacy. Furthermore cumbersome drug administration protocols had to be developed to overcome the pharmacokinetic obstacles associated with these agents. We therefore decided to investigate the effects of stably blocking angiogenesis by adopting a gene therapy based approach to ensure continuous expression of the antiangiogenic molecule.

The use of AAV5 and 8 vectors enabled us to express our antiangiogenic genes of interest in vivo rapidly, at high levels and most importantly stably. We have shown that tail vein injection of these vectors into immunodeficient mice, even at low doses, results in systemic protein expression as early as two weeks post transduction for AAV8 and 4 weeks for AAV5. Long term followup of these transduced mice shows persistent expression at high levels for atleast 6 months. The protocols for AAV production and purification developed collaboratively by members of our laboratory and Dr Davidoff's group at St Jude's Children's Research Hospital have resulted in easy and reproducible generation of high titer virus free of contaminants. This has allowed us to readily evaluate the antitumour effects of a range of molecules in murine tumour models. Furthermore the AAV8 production methodology has since been further optimised and scaled up to generate clinical grade vector (AAV8-FIX) for a clinical trial in patients with haemophilia B.

We have also optimised the method for producing lentiviral vectors which we have used very effectively to transduce a variety of haematological cell lines, some of which were historically difficult to transduce by other methods. We were able to generate high titer bicistronic lentiviral vectors which allowed us to express the antiangiogenic gene of interest as well as to use a reporter gene to track the transduced cells.

Our experiments with blocking the VEGF pathway by expressing the soluble form of one of its key receptors (sFlk-1) failed to demonstrate antitumour efficacy in AML or lymphoma models, despite sFlk-1 being expressed at supraphysiological levels. An alternative strategy of using an antibody against VEGF also failed to produce a

therapeutic effect in the same tumour models. Paradoxically there appeared to be a trend towards greater tumour progression in animals given the antibody than in controls, atleast at lower doses. There are several potential explanations for these results. Firstly it may be that these tumour models are too aggressive for antiangiogenesis to be effective as a single treatment modality and it may need to be combined with other therapies to see an inhibition of tumour growth. Secondly as we have described there are other receptors for VEGF which may still be able to signal upon binding with their ligand. However we have also shown no therapeutic effect with an anti-VEGF antibody which in theory should prevent binding of VEGF to any of its receptors. A third possibility is the compensatory upregulation of VEGF production by host stromal tissue, a phenomenon described previously by Davidoff (Davidoff et al., 2005b). Finally it is possible that in our tumour models VEGF may not be the critical angiogenic factor and therefore blockade of this pathway fails to produce a desired therapeutic effect as other pro-angiogenic cytokines play a more prominent role.

Previous clinical trials using matrix metalloproteinase inhibitors were largely unsuccessful at preventing tumour growth in several solid tumour types (Pavlaki and Zucker, 2003). However the use of the endogenous inhibitors of metalloproteinases (TIMPs) and in particular TIMP-3 had not previously been evaluated in AML or myeloma. We have shown for the first time significant antitumour effects when TIMP-3 was stably expressed within KMSBM myeloma cells in vivo both in terms of degree of tumour infiltration and prolongation of survival. In contrast TIMP-3 expression in HL-60 AML cells and ARH77 myeloma cells failed to result in any significant tumour regression. Possible explanations for these contrasting results include differences in the level of TIMP-3 expression between the different cell lines and potential differing roles played by the extracellular matrix in the different tumour models.

We have also shown impressive antileukaemic effects against both AML cell lines as well as primary leukaemia cells from poor risk AML patients by expressing human interferon-β continuously using AAV vectors in vivo. This is the first report of the therapeutic effects of hIFN-β against AML cells. Crucially we were able to demonstrate antitumour efficacy in vivo even at levels low enough to be clinically tolerable. In contrast recombinant human IFN-β failed to show any therapeutic effect against AML cells in vivo when administered either intraperitoneally or intravenously, despite adequate peak serum IFN-β levels being achieved by the intravenous route. Our data would suggest that maintenance of IFN-β levels is crucial for its antitumour effects and we would argue that

this could be achieved either by a gene therapy based approach or by the use of the pegylated form of recombinant hIFN-β. The main mechanisms of action of hIFN-β were found to antiproliferative and pro-apoptotic in this model although an indirect antiangiogenic effect could not be ruled out.

In summary we have developed xenograft models for AML, NHL and MM and have been able to show in our myeloma model significant antitumour efficacy in vivo when the antiangiogenic factor TIMP-3 was expressed within KMSBM myeloma cells. In addition we have convincingly demonstrated for the first time marked antileukaemic efficacy with human interferon-β against HL-60 cells and more importantly against primary leukaemic cells when this cytokine was expressed stably in vivo using AAV vector mediated gene transfer.

PUBLICATIONS ARISING FROM WORK PRESENTED IN THIS THESIS

Benjamin R, Khwaja A, Singh N, McIntosh J, Streck C, Ng CYC, Meager A, Wadhwa M, Davidoff AM, Nathwani AC (2007) Continuous delivery of human type I interferons(α/β) has significant activity against acute myeloid leukaemia cells in vitro and in a xenograft model. Blood 109(3) 1244-7

Rabin N, Kyriakou C, Coulton L, Gallagher OM, Buckle C, Benjamin R, Singh N, Glassford J, Otsuki T, Nathwani AC, Croucher PI, Yong K (2007) A new xenograft model of myeloma bone disease demonstrating the efficacy of human mesenchymal stem cells expressing osteoprotegerin by lentiviral gene transfer. Leukaemia (10) 2181-91

Kyriakou CA, Yong KL, Benjamin R, Pizzey A, Dogan A, Singh N, Davidoff AM, Nathwani AC (2006) Human mesenchymal stem cells (hMSCs) expressing truncated soluble vascular endothelial growth factor receptor (tsFlk-1) following lentiviral-mediated gene transfer inhibit growth of Burkitt's lymphoma in a murine model. J Gene Med 8(3) 253-64

REFERENCES

1994. Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. *The New England journal of medicine* 330:820-825.

Adamis, A.P., J.W. Miller, M.T. Bernal, D.J. D'Amico, J. Folkman, T.K. Yeo, and K.T. Yeo. 1994. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *American journal of ophthalmology* 118:445-450.

Aguayo, A., E. Estey, H. Kantarjian, T. Mansouri, C. Gidel, M. Keating, F. Giles, Z. Estrov, B. Barlogie, and M. Albitar. 1999. Cellular vascular endothelial growth factor is a predictor of outcome in patients with acute myeloid leukemia. *Blood* 94:3717-3721.

Aguayo, A., H. Kantarjian, T. Manshouri, C. Gidel, E. Estey, D. Thomas, C. Koller, Z. Estrov, S. O'Brien, M. Keating, E. Freireich, and M. Albitar. 2000. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. *Blood* 96:2240-2245.

Ahonen, M., R. Ala-Aho, A.H. Baker, S.J. George, R. Grenman, U. Saarialho-Kere, and V.M. Kahari. 2002. Antitumor activity and bystander effect of adenovirally delivered tissue inhibitor of metalloproteinases-3. *Molecular therapy : the journal of the American Society of Gene Therapy* 5:705-715.

Ahonen, M., A.H. Baker, and V.M. Kahari. 1998. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer research* 58:2310-2315.

Albini, A., C. Marchisone, F. Del Grosso, R. Benelli, L. Masiello, C. Tacchetti, M. Bono, M. Ferrantini, C. Rozera, M. Truini, F. Belardelli, L. Santi, and D.M. Noonan. 2000. Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: A gene therapy approach. *The American journal of pathology* 156:1381-1393.

Allan, N.C., S.M. Richards, and P.C. Shepherd. 1995. UK Medical Research Council randomised, multicentre trial of interferon-alpha n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. The UK Medical Research Council's Working Parties for Therapeutic Trials in Adult Leukaemia. *Lancet* 345:1392-1397.

Anand-Apte, B., L. Bao, R. Smith, K. Iwata, B.R. Olsen, B. Zetter, and S.S. Apte. 1996. A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 74:853-862.

Asahara, T., H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Magner, and J.M. Isner. 1999. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation research* 85:221-228.

Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J.M. Isner. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964-967.

Auricchio, A., E. O'Connor, M. Hildinger, and J.M. Wilson. 2001. A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* 4:372-374.

Baker, A.H., S.J. George, A.B. Zaltsman, G. Murphy, and A.C. Newby. 1999. Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. *British journal of cancer* 79:1347-1355.

Baker, A.H., A.B. Zaltsman, S.J. George, and A.C. Newby. 1998. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. *The Journal of clinical investigation* 101:1478-1487.

Baker, P.K., A.R. Pettitt, J.R. Slupsky, H.J. Chen, M.A. Glenn, M. Zuzel, and J.C. Cawley. 2002. Response of hairy cells to IFN-alpha involves induction of apoptosis through autocrine TNF-alpha and protection by adhesion. *Blood* 100:647-653.

Barille, S., C. Akhoundi, M. Collette, M.P. Mellerin, M.J. Rapp, J.L. Harousseau, R. Bataille, and M. Amiot. 1997. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. *Blood* 90:1649-1655.

Barille, S., R. Bataille, M.J. Rapp, J.L. Harousseau, and M. Amiot. 1999. Production of metalloproteinase-7 (matrilysin) by human myeloma cells and its potential involvement in metalloproteinase-2 activation. *J Immunol* 163:5723-5728.

Barr, P., P. Fu, H. Lazarus, D. Kane, H. Meyerson, P. Hartman, R. Reyes, R. Creger, K. Stear, M. Laughlin, W. Tse, and B. Cooper. 2007. Antiangiogenic activity of thalidomide in combination with fludarabine, carboplatin, and topotecan for high-risk acute myelogenous leukemia. *Leukemia & lymphoma* 48:1940-1949.

Bellamy, W.T., L. Richter, Y. Frutiger, and T.M. Grogan. 1999. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. *Cancer research* 59:728-733.

Bergers, G., R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, and D. Hanahan. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature cell biology* 2:737-744.

Bertolini, F., M. Paolucci, F. Peccatori, S. Cinieri, A. Agazzi, P.F. Ferrucci, E. Cocorocchio, A. Goldhirsch, and G. Martinelli. 1999. Angiogenic growth factors and endostatin in non-Hodgkin's lymphoma. *British journal of haematology* 106:504-509.

Borden, E.C., T.F. Hogan, and J.G. Voelkel. 1982. Comparative antiproliferative activity in vitro of natural interferons alpha and beta for diploid and transformed human cells. *Cancer research* 42:4948-4953.

Bouis, D., Y. Kusumanto, C. Meijer, N.H. Mulder, and G.A. Hospers. 2006. A review on pro- and anti-angiogenic factors as targets of clinical intervention. *Pharmacological research : the official journal of the Italian Pharmacological Society* 53:89-103.

Brown, L.F., K.T. Yeo, B. Berse, T.K. Yeo, D.R. Senger, H.F. Dvorak, and L. van de Water. 1992. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *The Journal of experimental medicine* 176:1375-1379.

Buckstein, R., R.S. Kerbel, Y. Shaked, R. Nayar, C. Foden, R. Turner, C.R. Lee, D. Taylor, L. Zhang, S. Man, S. Baruchel, D. Stempak, F. Bertolini, and M. Crump. 2006. High-Dose celecoxib and metronomic "low-dose" cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin's lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12:5190-5198.

Buckstein, R., R.M. Meyer, L. Seymour, J. Biagi, H. Mackay, S. Laurie, and E. Eisenhauer. 2007. Phase II testing of sunitinib: the National Cancer Institute of Canada Clinical Trials Group IND Program Trials IND.182-185. *Curr Oncol* 14:154-161.

Cavazzana-Calvo, M., Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A.2000. Gene therapy of human severe combined immunodeficiency (SCID)-XI disease. *Science* 288(5466)669-72.

Cavazzana-Calvo, M., Payen E, Negre O, Wang G, Hehir K, Fusil F, Down J, Denaro M, Brady T, Westerman K, Cavallesco R, Gillet-Legrand B, Caccavelli L, Sgarra R, Maouche-Chrétien L, Bernaudin F, Girot R, Dorazio R, Mulder GJ, Polack A, Bank A, Soulier J, Larghero J, Kabbara N, Dalle B, Gourmel B, Socie G, Chrétien S, Cartier N, Aubourg P, Fischer A, Cornetta K, Galacteros F, Beuzard Y, Gluckman E, Bushman F, Hacein-Bey-Abina S, and Leboulch P. 2010. Transfusion independence and HMGA2 activation after gene therapy of human thalassaemia. *Nature* 467(7313)318-22.

Chawla-Sarkar, M., D.W. Leaman, and E.C. Borden. 2001. Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. *Clinical cancer research : an official journal of the American Association for Cancer Research* 7:1821-1831.

Chen, Q., B. Gong, A.S. Mahmoud-Ahmed, A. Zhou, E.D. Hsi, M. Hussein, and A. Almasan. 2001. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. *Blood* 98:2183-2192.

Choi, K., M. Kennedy, A. Kazarov, J.C. Papadimitriou, and G. Keller. 1998. A common precursor for hematopoietic and endothelial cells. *Development* 125:725-732.

Clark, K.R., X. Liu, J.P. McGrath, and P.R. Johnson. 1999. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Human gene therapy* 10:1031-1039.

Cornelius, L.A., L.C. Nehring, E. Harding, M. Bolanowski, H.G. Welgus, D.K. Kobayashi, R.A. Pierce, and S.D. Shapiro. 1998. Matrix metalloproteinases generate angiostatin: effects on neovascularization. *J Immunol* 161:6845-6852.

Cozzolino, F., M. Torcia, M. Lucibello, L. Morbidelli, M. Ziche, J. Platt, S. Fabiani, J. Brett, and D. Stern. 1993. Interferon-alpha and interleukin 2 synergistically enhance basic fibroblast growth factor synthesis and induce release, promoting endothelial cell growth. *The Journal of clinical investigation* 91:2504-2512.

Dankbar, B., T. Padro, R. Leo, B. Feldmann, M. Kropff, R.M. Mesters, H. Serve, W.E. Berdel, and J. Kienast. 2000. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. *Blood* 95:2630-2636.

Davidoff, A.M., J.T. Gray, C.Y. Ng, Y. Zhang, J. Zhou, Y. Spence, Y. Bakar, and A.C. Nathwani. 2005a. Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. *Molecular therapy : the journal of the American Society of Gene Therapy* 11:875-888.

Davidoff, A.M., A.C. Nathwani, W.W. Spurbeck, C.Y. Ng, J. Zhou, and E.F. Vanin. 2002. rAAV-mediated long-term liver-generated expression of an angiogenesis inhibitor can restrict renal tumor growth in mice. *Cancer research* 62:3077-3083.

Davidoff, A.M., C.Y. Ng, S. Sleep, J. Gray, S. Azam, Y. Zhao, J.H. McIntosh, M. Karimipoor, and A.C. Nathwani. 2004. Purification of recombinant adeno-

associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. *Journal of virological methods* 121:209-215.

Davidoff, A.M., C.Y. Ng, Y. Zhang, C.J. Streck, S.J. Mabry, S.H. Barton, T. Baudino, J. Zhou, R.S. Kerbel, E.F. Vanin, and A.C. Nathwani. 2005b. Careful decoy receptor titering is required to inhibit tumor angiogenesis while avoiding adversely altering VEGF bioavailability. *Molecular therapy : the journal of the American Society of Gene Therapy* 11:300-310.

Dias, S., K. Hattori, B. Heissig, Z. Zhu, Y. Wu, L. Witte, D.J. Hicklin, M. Tateno, P. Bohlen, M.A. Moore, and S. Rafii. 2001. Inhibition of both paracrine and autocrine VEGF/ VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias. *Proceedings of the National Academy of Sciences of the United States of America* 98:10857-10862.

Dias, S., K. Hattori, Z. Zhu, B. Heissig, M. Choy, W. Lane, Y. Wu, A. Chadburn, E. Hyjek, M. Gill, D.J. Hicklin, L. Witte, M.A. Moore, and S. Rafii. 2000. Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *The Journal of clinical investigation* 106:511-521.

Dickson, P.V., J.B. Hamner, C.J. Streck, C.Y. Ng, M.B. McCarville, C. Calabrese, R.J. Gilbertson, C.F. Stewart, C.M. Wilson, M.W. Gaber, L.M. Pfeffer, S.X. Skapek, A.C. Nathwani, and A.M. Davidoff. 2007. Continuous delivery of IFN-beta promotes sustained maturation of intratumoral vasculature. *Molecular cancer research : MCR* 5:531-542.

Dinney, C.P., D.R. Bielenberg, P. Perrotte, R. Reich, B.Y. Eve, C.D. Bucana, and I.J. Fidler. 1998. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer research* 58:808-814.

Dong, Z., G. Greene, C. Pettaway, C.P. Dinney, I. Eue, W. Lu, C.D. Bucana, M.D. Balbay, D. Bielenberg, and I.J. Fidler. 1999. Suppression of angiogenesis, tumorigenicity, and metastasis by human prostate cancer cells engineered to produce interferon-beta. *Cancer research* 59:872-879.

Doussis-Anagnostopoulou, I.A., K.L. Talks, H. Turley, P. Debnam, D.C. Tan, G. Mariatos, V. Gorgoulis, C. Kittas, and K.C. Gatter. 2002. Vascular endothelial growth factor (VEGF) is expressed by neoplastic Hodgkin-Reed-Sternberg cells in Hodgkin's disease. *The Journal of pathology* 197:677-683.

Duggan, D.B., M.T. Santarelli, K. Zamkoff, S. Lichtman, J. Ellerton, R. Cooper, B. Poiesz, J.R. Anderson, C.D. Bloomfield, B.A. Peterson, and et al. 1992. A phase II study of recombinant interleukin-2 with or without recombinant interferon-beta in non-Hodgkin's lymphoma. A study of the Cancer and Leukemia Group B. *Journal of immunotherapy : official journal of the Society for Biological Therapy* 12:115-122.

Dvorak, H.F., J.A. Nagy, D. Feng, L.F. Brown, and A.M. Dvorak. 1999. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Current topics in microbiology and immunology* 237:97-132.

Einhorn, S., J.O. Fernberg, D. Grander, and R. Lewensohn. 1988. Interferon exerts a cytotoxic effect on primary human myeloma cells. *European journal of cancer & clinical oncology* 24:1505-1510.

Fabra, A., M. Nakajima, C.D. Bucana, and I.J. Fidler. 1992. Modulation of the invasive phenotype of human colon carcinoma cells by organ specific fibroblasts of nude mice. *Differentiation; research in biological diversity* 52:101-110.

Favier, B., A. Alam, P. Barron, J. Bonnin, P. Laboudie, P. Fons, M. Mandron, J.P. Herault, G. Neufeld, P. Savi, J.M. Herbert, and F. Bono. 2006. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood* 108:1243-1250.

Feldman, D., A.L. Goldstein, D.C. Cox, and P.M. Grimley. 1988. Cultured human endothelial cells treated with recombinant leukocyte A interferon. Tubuloreticular inclusion formation, antiproliferative effect, and 2',5' oligoadenylate synthetase induction. *Laboratory investigation; a journal of technical methods and pathology* 58:584-589.

Ferrara, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocrine reviews* 25:581-611.

Fiedler, W., U. Graeven, S. Ergun, S. Verago, N. Kilic, M. Stockschlader, and D.K. Hossfeld. 1997. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. *Blood* 89:1870-1875.

Fiedler, W., R. Mesters, H. Tinnefeld, S. Loges, P. Staib, U. Duhrsen, M. Flasshove, O.G. Ottmann, W. Jung, F. Cavalli, R. Kuse, J. Thomalla, H. Serve, A.M. O'Farrell, M. Jacobs, N.M. Brega, P. Scigalla, D.K. Hossfeld, and W.E. Berdel. 2003. A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. *Blood* 102:2763-2767.

Flavell, J.R., K.R. Baumforth, D.M. Williams, M. Lukesova, J. Madarova, V. Noskova, J. Prochazkova, D. Lowe, Z. Kolar, P.G. Murray, and P.N. Nelson. 2000. Expression of the matrix metalloproteinase 9 in Hodgkin's disease is independent of EBV status. *Molecular pathology : MP* 53:145-149.

Folkman, J. 1990. What is the evidence that tumors are angiogenesis dependent? *Journal of the National Cancer Institute* 82:4-6.

Fong, G.H., J. Rossant, M. Gertsenstein, and M.L. Breitman. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376:66-70.

Foss, H.D., I. Araujo, G. Demel, H. Klotzbach, M. Hummel, and H. Stein. 1997. Expression of vascular endothelial growth factor in lymphomas and Castleman's disease. *The Journal of pathology* 183:44-50.

Fusetti, L., G. Pruneri, A. Gobbi, C. Rabascio, N. Carboni, F. Peccatori, G. Martinelli, and F. Bertolini. 2000. Human myeloid and lymphoid malignancies in the non-obese diabetic/severe combined immunodeficiency mouse model: frequency of apoptotic cells in solid tumors and efficiency and speed of engraftment correlate with vascular endothelial growth factor production. *Cancer research* 60:2527-2534.

Ganjoo, K.N., C.S. An, M.J. Robertson, L.I. Gordon, J.A. Sen, J. Weisenbach, S. Li, E.A. Weller, A. Orazi, and S.J. Horning. 2006. Rituximab, bevacizumab and CHOP (RA-CHOP) in untreated diffuse large B-cell lymphoma: safety, biomarker and pharmacokinetic analysis. *Leukemia & lymphoma* 47:998-1005.

Gering, M., A.R. Rodaway, B. Gottgens, R.K. Patient, and A.R. Green. 1998. The SCL gene specifies haemangioblast development from early mesoderm. *The EMBO journal* 17:4029-4045.

Gertz, M.A., L.A. Kalish, R.A. Kyle, R.G. Hahn, D.C. Tormey, and M.M. Oken. 1995. Phase III study comparing vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD) chemotherapy with VAD plus recombinant interferon alfa-2 in refractory or relapsed multiple myeloma. An Eastern Cooperative Oncology Group study. *American journal of clinical oncology* 18:475-480.

Giannopoulos, K., A. Dmoszynska, M. Kowal, E. Wasik-Szczepanek, A. Bojarska-Junak, J. Rolinski, H. Dohner, S. Stilgenbauer, and L. Bullinger. 2009. Thalidomide exerts distinct molecular antileukemic effects and combined thalidomide/fludarabine therapy is clinically effective in high-risk chronic lymphocytic leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 23:1771-1778.

Glimelius, I., A. Edstrom, M. Fischer, G. Nilsson, C. Sundstrom, D. Molin, R.M. Amini, and G. Enblad. 2005. Angiogenesis and mast cells in Hodgkin lymphoma. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 19:2360-2362.

Gohji, K., I.J. Fidler, R. Tsan, R. Radinsky, A.C. von Eschenbach, T. Tsuruo, and M. Nakajima. 1994. Human recombinant interferons-beta and -gamma decrease gelatinase production and invasion by human KG-2 renal-carcinoma cells. *International journal of cancer. Journal international du cancer* 58:380-384.

Goldstone, A.H., A.K. Burnett, K. Wheatley, A.G. Smith, R.M. Hutchinson, and R.E. Clark. 2001. Attempts to improve treatment outcomes in acute myeloid leukemia (AML) in older patients: the results of the United Kingdom Medical Research Council AML11 trial. *Blood* 98:1302-1311.

Gomez, D., and N.C. Reich. 2003. Stimulation of primary human endothelial cell proliferation by IFN. *J Immunol* 170:5373-5381.

Gomez, D.E., D.F. Alonso, H. Yoshiji, and U.P. Thorgeirsson. 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *European journal of cell biology* 74:111-122.

Goodbourn, S., L. Didcock, and R.E. Randall. 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *The Journal of general virology* 81:2341-2364.

Gray, S.J., V. Matagne, L. Bachaboina, S. Yadav, S.R. Ojeda, R.J. Samulski. 2011 Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther* 19(6) 1058-69.

Hanemaaijer, R., J.H. Verheijen, T.M. Maguire, H. Visser, K. Toet, E. McDermott, N. O'Higgins, and M.J. Duffy. 2000. Increased gelatinase-A and gelatinase-B activities in malignant vs. benign breast tumors. *International journal of cancer. Journal international du cancer* 86:204-207.

Hayakawa, T., K. Yamashita, E. Ohuchi, and A. Shinagawa. 1994. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *Journal of cell science* 107 (Pt 9):2373-2379.

Hayakawa, T., K. Yamashita, K. Tanzawa, E. Uchijima, and K. Iwata. 1992. Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS letters* 298:29-32.

Hazar, B., S. Paydas, S. Zorludemir, B. Sahin, and I. Tuncer. 2003. Prognostic significance of microvessel density and vascular endothelial growth factor (VEGF) expression in non-Hodgkin's lymphoma. *Leukemia & lymphoma* 44:2089-2093.

Hendrix, M.J., E.A. Seftor, T.M. Grogan, R.E. Seftor, E.M. Hersh, E.A. Boyse, L.A. Liotta, W. Stetler-Stevenson, and C.G. Ray. 1992. Expression of type IV collagenase correlates with the invasion of human lymphoblastoid cell lines and pathogenesis in SCID mice. *Molecular and cellular probes* 6:59-65.

Herren, B., B. Levkau, E.W. Raines, and R. Ross. 1998. Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence

for a role for caspases and metalloproteinases. *Molecular biology of the cell* 9:1589-1601.

Hicklin, D.J., and L.M. Ellis. 2005. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23:1011-1027.

Ho, C.L., L.F. Sheu, and C.Y. Li. 2002. Immunohistochemical expression of basic fibroblast growth factor, vascular endothelial growth factor, and their receptors in stage IV non-Hodgkin lymphoma. *Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry* 10:316-321.

Hofmockel, G., W. Langer, M. Theiss, A. Gruss, and H.G. Frohmuller. 1996. Immunochemotherapy for metastatic renal cell carcinoma using a regimen of interleukin-2, interferon-alpha and 5-fluorouracil. *The Journal of urology* 156:18-21.

Howe, S.J., M.R. Mansour, K. Schwarzwaelder, C. Bartholomae, M. Hubank, H. Kempski, M.H. Brugman, K. Pike-Overzet, S.J. Chatters, D. de Ridder, K.C. Gilmour, S. Adams, S.I. Thornhill, K.L. Parsley, F.J. Staal, R.E. Gale, D.C. Linch, J. Bayford, L. Brown, M. Quaye, C. Kinnon, P.Ancliff , D.K. Webb, M. Schmidt, C. von Kalle, H.B. Gaspar, A.J. Thrasher. 2008. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis floowing gene therapy of SCID-XI patients. *J Clin Invest* 118(9)3143-50.

Hussong, J.W., G.M. Rodgers, and P.J. Shami. 2000. Evidence of increased angiogenesis in patients with acute myeloid leukemia. *Blood* 95:309-313.

Igreja, C., M. Courinha, A.S. Cachaco, T. Pereira, J. Cabecadas, M.G. da Silva, and S. Dias. 2007. Characterization and clinical relevance of circulating and biopsy-derived endothelial progenitor cells in lymphoma patients. *Haematologica* 92:469-477.

Ivanoff, A., J. Ivanoff, K. Hultenby, and K.G. Sundqvist. 1999. Infiltrative capacity of T leukemia cell lines: a distinct functional property coupled to expression of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1). *Clinical & experimental metastasis* 17:695-711.

Izawa, J.I., P. Sweeney, P. Perrotte, D. Kedar, Z. Dong, J.W. Slaton, T. Karashima, K. Inoue, W.F. Benedict, and C.P. Dinney. 2002. Inhibition of tumorigenicity and metastasis of human bladder cancer growing in athymic mice by interferon-beta gene therapy results partially from various antiangiogenic effects including endothelial cell apoptosis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 8:1258-1270.

Janowska-Wieczorek, A., L.A. Marquez, A. Matsuzaki, H.R. Hashmi, L.M. Larratt, L.M. Boshkov, A.R. Turner, M.C. Zhang, D.R. Edwards, and A.E. Kossakowska. 1999. Expression of matrix metalloproteinases (MMP-2 and -9) and tissue inhibitors of metalloproteinases (TIMP-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. *British journal of haematology* 105:402-411.

Johns, T.G., I.R. Mackay, K.A. Callister, P.J. Hertzog, R.J. Devenish, and A.W. Linnane. 1992. Antiproliferative potencies of interferons on melanoma cell lines and xenografts: higher efficacy of interferon beta. *Journal of the National Cancer Institute* 84:1185-1190.

Jonasch, E., and F.G. Haluska. 2001. Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities. *The oncologist* 6:34-55.

Jorgensen, J.M., F.B. Sorensen, K. Bendix, J.L. Nielsen, M.L. Olsen, A.M. Funder, and F. d'Amore. 2007. Angiogenesis in non-Hodgkin's lymphoma: clinico-pathological correlations and prognostic significance in specific subtypes. *Leukemia & lymphoma* 48:584-595.

Karp, J.E., I. Gojo, R. Pili, C.D. Gocke, J. Greer, C. Guo, D. Qian, L. Morris, M. Tidwell, H. Chen, and J. Zwiebel. 2004. Targeting vascular endothelial growth factor for relapsed and refractory adult acute myelogenous leukemias: therapy with sequential 1-beta-d-arabinofuranosylcytosine, mitoxantrone, and bevacizumab. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10:3577-3585.

Kato, N., A. Nawa, K. Tamakoshi, F. Kikkawa, N. Suganuma, T. Okamoto, S. Goto, Y. Tomoda, M. Hamaguchi, and M. Nakajima. 1995. Suppression of gelatinase production with decreased invasiveness of choriocarcinoma cells by human recombinant interferon beta. *American journal of obstetrics and gynecology* 172:601-606.

Kaufmann, H., M. Raderer, S. Wohrer, A. Puspok, A. Bankier, C. Zielinski, A. Chott, and J. Drach. 2004. Antitumor activity of rituximab plus thalidomide in patients with relapsed/refractory mantle cell lymphoma. *Blood* 104:2269-2271.

Kchour, G., M. Tarhini, N. Sharifi, R. Farid, A.R. Khooei, A. Shirdel, J.T. Afshari, A. Sadeghian, Z. Otrock, O. Hermine, M. El-Sabban, and A. Bazarbachi. 2008. Increased microvessel density in involved organs from patients with HTLV-I associated adult T cell leukemia lymphoma. *Leukemia & lymphoma* 49:265-270.

Kerbel, R.S. 1991. Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. *BioEssays : news and reviews in molecular, cellular and developmental biology* 13:31-36.

Koomagi, R., F. Zintl, A. Sauerbrey, and M. Volm. 2001. Vascular endothelial growth factor in newly diagnosed and recurrent childhood acute lymphoblastic leukemia as measured by real-time quantitative polymerase chain reaction. *Clinical cancer research : an official journal of the American Association for Cancer Research* 7:3381-3384.

Korkolopoulou, P., I. Thymara, N. Kavantzas, T.P. Vassilakopoulos, M.K. Angelopoulou, S.I. Kokoris, E.M. Dimitriadou, M.P. Siakantaris, K. Anargyrou, P. Panayiotidis, A. Tsenga, A. Androulaki, I.A. Doussis-Anagnostopoulou, E. Patsouris, and G.A. Pangalis. 2005. Angiogenesis in Hodgkin's lymphoma: a morphometric approach in 286 patients with prognostic implications. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 19:894-900.

Koster, A., J.H. van Krieken, M.A. Mackenzie, M. Schraders, G.F. Borm, J.A. van der Laak, W. Leenders, K. Hebeda, and J.M. Raemaekers. 2005. Increased vascularization predicts favorable outcome in follicular lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11:154-161.

Kreuter, M., K. Woelke, R. Bieker, C. Schliemann, M. Steins, T. Buechner, W.E. Berdel, and R.M. Mesters. 2006. Correlation of neuropilin-1 overexpression to survival in acute myeloid leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 20:1950-1954.

Kuittinen, O., E.R. Savolainen, P. Koistinen, M. Mottonen, and T. Turpeenniemi-Hujanen. 2001. MMP-2 and MMP-9 expression in adult and childhood acute lymphatic leukemia (ALL). *Leukemia research* 25:125-131.

Kuittinen, O., E.R. Savolainen, P. Koistinen, and T. Turpeenniemi-Hujanen. 1999. Gelatinase A and B (MMP-2, MMP-9) in leukaemia MMP-2 may indicate a good prognosis in AML. *Anticancer research* 19:4395-4400.

Kumar, S., R. Fonseca, A. Dispenzieri, M.Q. Lacy, J.A. Lust, T.E. Witzig, M.A. Gertz, R.A. Kyle, P.R. Greipp, and S.V. Rajkumar. 2002. Bone marrow angiogenesis in multiple myeloma: effect of therapy. *British journal of haematology* 119:665-671.

Kumar, S., M.A. Gertz, A. Dispenzieri, M.Q. Lacy, L.A. Wellik, R. Fonseca, J.A. Lust, T.E. Witzig, R.A. Kyle, P.R. Greipp, and S.V. Rajkumar. 2004. Prognostic value of bone marrow angiogenesis in patients with multiple myeloma undergoing high-dose therapy. *Bone marrow transplantation* 34:235-239.

Leandersson, T., and E. Lundgren. 1980. Antiproliferative effect on interferon on a Burkitt's lymphoma cell line. *Experimental cell research* 130:421-426.

Leco, K.J., R. Khokha, N. Pavloff, S.P. Hawkes, and D.R. Edwards. 1994. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *The Journal of biological chemistry* 269:9352-9360.

Lee, W.H., F.H. Liu, Y.L. Lee, and H.M. Huang. 2010. Interferon-alpha induces the growth inhibition of human T-cell leukaemia line Jurkat through p38alpha and p38beta. *Journal of biochemistry* 147:645-650.

Lengyel, P. 1993. Tumor-suppressor genes: news about the interferon connection. *Proceedings of the National Academy of Sciences of the United States of America* 90:5893-5895.

Levine, A.M., A. Tulpule, D.I. Quinn, G. Gorospe, 3rd, D.L. Smith, L. Hornor, W.D. Boswell, B.M. Espina, S.G. Groshen, R. Masood, and P.S. Gill. 2006. Phase I study of antisense oligonucleotide against vascular endothelial growth factor: decrease in plasma vascular endothelial growth factor with potential clinical efficacy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24:1712-1719.

Liberati, A.M., S. Cinieri, M.G. Senatore, M.G. Portuesi, V. De Angelis, F. Di Clemente, M. Schippa, A. Ferrajoli, S. Arzano, and P. Berruto. 1990a. Phase I-II trial on natural beta interferon in chemoresistant and relapsing multiple myeloma. *Haematologica* 75:436-442.

Liberati, A.M., M. Fizzotti, F. Di Clemente, M. Senatore, P. Berruto, B. Falini, M.F. Martelli, and F. Grignani. 1990b. Response to intermediate and standard doses of IFN-beta in hairy-cell leukaemia. *Leukemia research* 14:779-784.

Liberati, A.M., M. Horisberger, M. Schippa, F. Di Clemente, M. Fizzotti, S. Filippo, M.G. Proietti, S. Arzano, P. Berruto, L. Palmisano, and et al. 1991. Biochemical and immunological responses of hairy cell leukemia patients to interferon beta. *Cancer immunology, immunotherapy : CII* 34:115-122.

Lin, L.I., D.T. Lin, C.J. Chang, C.Y. Lee, J.L. Tang, and H.F. Tien. 2002. Marrow matrix metalloproteinases (MMPs) and tissue inhibitors of MMP in acute leukaemia: potential role of MMP-9 as a surrogate marker to monitor leukaemic status in patients with acute myelogenous leukaemia. *British journal of haematology* 117:835-841.

Maguire, A.M., Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zelenaia O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA and J. Bennett. 2008. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* 358(21)2240-8.

Mainou-Fowler, T., B. Angus, S. Miller, S.J. Proctor, P.R. Taylor, and K.M. Wood. 2006. Micro-vessel density and the expression of vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PdEGF) in classical Hodgkin lymphoma (HL). *Leukemia & lymphoma* 47:223-230.

Mandelli, F., G. Avvisati, S. Amadori, M. Boccadoro, A. Gernone, V.M. Lauta, F. Marmont, M.T. Petrucci, M. Tribalto, M.L. Vegna, and et al. 1990. Maintenance treatment with recombinant interferon alfa-2b in patients with multiple myeloma responding to conventional induction chemotherapy. *The New England journal of medicine* 322:1430-1434.

Marth, C., M.V. Cronauer, W. Doppler, D. Ofner, A. Ullrich, and G. Daxenbichler. 1992. Effects of interferons on the expression of the proto-oncogene HER-2 in human ovarian carcinoma cells. *International journal of cancer. Journal international du cancer* 50:64-68.

Meager, A. 2004. Assays for antiviral activity. *Methods Mol Biol* 249:121-134.

Medinger, M., and K. Mross. 2010. Clinical trials with anti-angiogenic agents in hematological malignancies. *Journal of angiogenesis research* 2:10.

Minasian, L.M., R.J. Motzer, L. Gluck, M. Mazumdar, V. Vlamis, and S.E. Krown. 1993. Interferon alfa-2a in advanced renal cell carcinoma: treatment results and survival in 159 patients with long-term follow-up. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 11:1368-1375.

Minischetti, M., A. Vacca, D. Ribatti, M. Iurlaro, R. Ria, A. Pellegrino, G. Gasparini, and A.F. Dammacco. 2000. TNP-470 and recombinant human interferon-alpha2a inhibit angiogenesis synergistically. *British journal of haematology* 109:829-837.

Monestiroli, S., P. Mancuso, A. Burlini, G. Pruneri, C. Dell'Agnola, A. Gobbi, G. Martinelli, and F. Bertolini. 2001. Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. *Cancer research* 61:4341-4344.

Montini, E., Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C and Naldini L. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 24(6)687-96.

Morrison, B.H., J.A. Bauer, D.V. Kalvakolanu, and D.J. Lindner. 2001. Inositol hexakisphosphate kinase 2 mediates growth suppressive and apoptotic effects of interferon-beta in ovarian carcinoma cells. *The Journal of biological chemistry* 276:24965-24970.

Munshi, N.C., and C. Wilson. 2001. Increased bone marrow microvessel density in newly diagnosed multiple myeloma carries a poor prognosis. *Seminars in oncology* 28:565-569.

Murphy, G., V. Knauper, M.H. Lee, A. Amour, J.R. Worley, M. Hutton, S. Atkinson, M. Rapti, and R. Williamson. 2003. Role of TIMPs (tissue inhibitors of metalloproteinases) in pericellular proteolysis: the specificity is in the detail. *Biochemical Society symposium* 65-80.

Nagase, H., and J.F. Woessner, Jr. 1999. Matrix metalloproteinases. *The Journal of biological chemistry* 274:21491-21494.

Nathwani, A.C., A. Davidoff, H. Hanawa, J.F. Zhou, E.F. Vanin, and A.W. Nienhuis. 2001. Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. *Blood* 97:1258-1265.

Nathwani, A.C., J.T. Gray, C.Y. Ng, J. Zhou, Y. Spence, S.N. Waddington, E.G. Tuddenham, G. Kemball-Cook, J. McIntosh, M. Boon-Spijker, K. Mertens, A.M.

Davidoff. 2006. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 107(7)2653-61.

Nathwani, A.C., J.T. Gray, J. McIntosh, C.Y. Ng, J. Zhou, Y. Spence, M. Cochrane, E. Gray, E.G. Tuddenham, A.M. Davidoff. 2007. Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* 109(4)1414-21.

Negrier, S., B. Escudier, C. Lasset, J.Y. Douillard, J. Savary, C. Chevreau, A. Ravaud, A. Mercatello, J. Peny, M. Mousseau, T. Philip, and T. Tursz. 1998. Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie. *The New England journal of medicine* 338:1272-1278.

Nishizuka, I., Y. Ichikawa, T. Ishikawa, M. Kamiyama, S. Hasegawa, N. Momiyama, K. Miyazaki, and H. Shimada. 2001. Matrilysin stimulates DNA synthesis of cultured vascular endothelial cells and induces angiogenesis in vivo. *Cancer letters* 173:175-182.

Ohtsuki, T., Y. Yawata, H. Wada, T. Sugihara, M. Mori, and M. Namba. 1989. Two human myeloma cell lines, amylase-producing KMS-12-PE and amylase-non-producing KMS-12-BM, were established from a patient, having the same chromosome marker, t(11;14)(q13;q32). *British journal of haematology* 73:199-204.

Oken, M.M., T. Leong, R.E. Lenhard, Jr., P.R. Greipp, N.E. Kay, B. Van Ness, R.M. Keimowitz, and R.A. Kyle. 1999. The addition of interferon or high dose cyclophosphamide to standard chemotherapy in the treatment of patients with multiple myeloma: phase III Eastern Cooperative Oncology Group Clinical Trial EST 9486. *Cancer* 86:957-968.

Osterborg, A., M. Bjorkholm, M. Bjoreman, G. Brenning, K. Carlson, F. Celsing, G. Gahrton, G. Grimfors, H. Gyllenhammar, R. Hast, and et al. 1993. Natural interferon-alpha in combination with melphalan/prednisone versus melphalan/prednisone in the treatment of multiple myeloma stages II and III: a randomized study from the Myeloma Group of Central Sweden. *Blood* 81:1428-1434.

Ozawa, S., H. Shinohara, H.O. Kanayama, C.J. Bruns, C.D. Bucana, L.M. Ellis, D.W. Davis, and I.J. Fidler. 2001. Suppression of angiogenesis and therapy of human colon cancer liver metastasis by systemic administration of interferon-alpha. *Neoplasia* 3:154-164.

Pammer, J., C. Reinisch, P. Birner, K. Pogoda, M. Sturzl, and E. Tschachler. 2006. Interferon-alpha prevents apoptosis of endothelial cells after short-term exposure but induces replicative senescence after continuous stimulation. *Laboratory investigation; a journal of technical methods and pathology* 86:997-1007.

Passalidou, E., M. Stewart, M. Trivella, G. Steers, G. Pillai, A. Dogan, I. Leigh, C. Hatton, A. Harris, K. Gatter, and F. Pezzella. 2003. Vascular patterns in reactive lymphoid tissue and in non-Hodgkin's lymphoma. *British journal of cancer* 88:553-559.

Passam, F.H., A. Sfiridaki, C. Pappa, D. Kyriakou, E. Petreli, P.A. Roussou, and M.G. Alexandrakis. 2008. Angiogenesis-related growth factors and cytokines in the serum of patients with B non-Hodgkin lymphoma; relation to clinical features and response to treatment. *International journal of laboratory hematology* 30:17-25.

Pavlaki, M., and S. Zucker. 2003. Matrix metalloproteinase inhibitors (MMPIs): the beginning of phase I or the termination of phase III clinical trials. *Cancer metastasis reviews* 22:177-203.

Peest, D., H. Deicher, R. Coldewey, R. Leo, R. Bartl, H. Bartels, H.J. Braun, W. Fett, J.T. Fischer, B. Gobel, and et al. 1995. A comparison of polychemotherapy and melphalan/prednisone for primary remission induction, and interferon-alpha for maintenance treatment, in multiple myeloma. A prospective trial of the German Myeloma Treatment Group. *Eur J Cancer* 31A:146-151.

Pennanen, H., O. Kuittinen, Y. Soini, and T. Turpeenniemi-Hujanen. 2008. Prognostic significance of p53 and matrix metalloproteinase-9 expression in follicular lymphoma. *European journal of haematology* 81:289-297.

Perez-Atayde, A.R., S.E. Sallan, U. Tedrow, S. Connors, E. Allred, and J. Folkman. 1997. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. *The American journal of pathology* 150:815-821.

Podar, K., and K.C. Anderson. 2005. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. *Blood* 105:1383-1395.

Podar, K., Y.T. Tai, F.E. Davies, S. Lentzsch, M. Sattler, T. Hideshima, B.K. Lin, D. Gupta, Y. Shima, D. Chauhan, C. Mitsiades, N. Raje, P. Richardson, and K.C. Anderson. 2001. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. *Blood* 98:428-435.

Podar, K., G. Tonon, M. Sattler, Y.T. Tai, S. Legouill, H. Yasui, K. Ishitsuka, S. Kumar, R. Kumar, L.N. Pandite, T. Hideshima, D. Chauhan, and K.C. Anderson. 2006. The small-molecule VEGF receptor inhibitor pazopanib (GW786034B) targets both tumor and endothelial cells in multiple myeloma. *Proceedings of the National Academy of Sciences of the United States of America* 103:19478-19483.

Qi, J.H., Q. Ebrahem, N. Moore, G. Murphy, L. Claesson-Welsh, M. Bond, A. Baker, and B. Anand-Apte. 2003. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nature medicine* 9:407-415.

Quesada, J.R., J. Reuben, J.T. Manning, E.M. Hersh, and J.U. Gutterman. 1984. Alpha interferon for induction of remission in hairy-cell leukemia. *The New England journal of medicine* 310:15-18.

Rabin, N., C. Kyriakou, L. Coulton, O.M. Gallagher, C. Buckle, R. Benjamin, N. Singh, J. Glassford, T. Otsuki, A.C. Nathwani, P.I. Croucher, and K.L. Yong. 2007. A new xenograft model of myeloma bone disease demonstrating the efficacy of human mesenchymal stem cells expressing osteoprotegerin by lentiviral gene transfer. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 21:2181-2191.

Rajkumar, S.V., E. Blood, D. Vesole, R. Fonseca, and P.R. Greipp. 2006. Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24:431-436.

Rajkumar, S.V., R. Fonseca, T.E. Witzig, M.A. Gertz, and P.R. Greipp. 1999. Bone marrow angiogenesis in patients achieving complete response after stem cell transplantation for multiple myeloma. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 13:469-472.

Rajkumar, S.V., T. Leong, P.C. Roche, R. Fonseca, A. Dispenzieri, M.Q. Lacy, J.A. Lust, T.E. Witzig, R.A. Kyle, M.A. Gertz, and P.R. Greipp. 2000. Prognostic value of

bone marrow angiogenesis in multiple myeloma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 6:3111-3116.

Rao, K.V. 2007. Lenalidomide in the treatment of multiple myeloma. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* 64:1799-1807.

Ribas, C., G.W. Colleoni, M.R. Silva, M.J. Carregoza, and J.O. Bordin. 2004. Prognostic significance of vascular endothelial growth factor immunoexpression in the context of adverse standard prognostic factors in multiple myeloma. *European journal of haematology* 73:311-317.

Ribatti, D., A. Vacca, A. Marzullo, B. Nico, R. Ria, L. Roncali, and F. Dammacco. 2000. Angiogenesis and mast cell density with tryptase activity increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas. *International journal of cancer. Journal international du cancer* 85:171-175.

Roboz, G.J., F.J. Giles, A.F. List, J.E. Cortes, R. Carlin, M. Kowalski, S. Bilic, E. Masson, M. Rosamilia, M.W. Schuster, D. Laurent, and E.J. Feldman. 2006. Phase 1 study of PTK787/ZK 222584, a small molecule tyrosine kinase receptor inhibitor, for the treatment of acute myeloid leukemia and myelodysplastic syndrome. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 20:952-957.

Roccaro, A.M., T. Hideshima, N. Raje, S. Kumar, K. Ishitsuka, H. Yasui, N. Shiraishi, D. Ribatti, B. Nico, A. Vacca, F. Dammacco, P.G. Richardson, and K.C. Anderson. 2006. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. *Cancer research* 66:184-191.

Romerio, F., and D. Zella. 2002. MEK and ERK inhibitors enhance the anti-proliferative effect of interferon-alpha2b. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 16:1680-1682.

Rosenblum, M.G., W.K. Yung, P.J. Kelleher, F. Ruzicka, P.A. Steck, and E.C. Borden. 1990. Growth inhibitory effects of interferon-beta but not interferon-alpha on human glioma cells: correlation of receptor binding, 2',5'-oligoadenylate synthetase and protein kinase activity. *Journal of interferon research* 10:141-151.

Rosewicz, S., K. Detjen, A. Scholz, and Z. von Marschall. 2004. Interferon-alpha: regulatory effects on cell cycle and angiogenesis. *Neuroendocrinology* 80 Suppl 1:85-93.

Rousseau, S., F. Houle, H. Kotanides, L. Witte, J. Waltenberger, J. Landry, and J. Huot. 2000. Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. *The Journal of biological chemistry* 275:10661-10672.

Rueda, A., D. Olmos, V. Villareal, E. Torres, B.I. Pajares, and E. Alba. 2007. Elevated vascular endothelial growth factor pretreatment levels are correlated with the tumor burden in Hodgkin lymphoma and continue to be elevated in prolonged complete remission. *Clinical lymphoma & myeloma* 7:400-405.

Ruszczak, Z., M. Detmar, E. Imcke, and C.E. Orfanos. 1990. Effects of rIFN alpha, beta, and gamma on the morphology, proliferation, and cell surface antigen expression of human dermal microvascular endothelial cells in vitro. *The Journal of investigative dermatology* 95:693-699.

Sakata, K., M. Satoh, M. Someya, H. Asanuma, H. Nagakura, A. Oouchi, K. Nakata, K. Kogawa, K. Koito, M. Hareyama, and T. Himi. 2004. Expression of matrix metalloproteinase 9 is a prognostic factor in patients with non-Hodgkin lymphoma. *Cancer* 100:356-365.

Salmon, P., J.Y. Le Cotonnec, A. Galazka, A. Abdul-Ahad, and A. Darragh. 1996. Pharmacokinetics and pharmacodynamics of recombinant human interferon-beta in healthy male volunteers. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 16:759-764.

Salmon, S.E., J.J. Crowley, T.M. Grogan, P. Finley, R.P. Pugh, and B. Barlogie. 1994. Combination chemotherapy, glucocorticoids, and interferon alfa in the treatment of multiple myeloma: a Southwest Oncology Group study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 12:2405-2414.

Salven, P., K. Anttonen, H. Repo, H. Joensuu, and A. Orpana. 2001. Endotoxins induce and interferon alpha suppresses vascular endothelial growth factor (VEGF) production in human peripheral blood mononuclear cells. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 15:1318-1320.

Salven, P., L. Teerenhovi, and H. Joensuu. 1997. A high pretreatment serum vascular endothelial growth factor concentration is associated with poor outcome in non-Hodgkin's lymphoma. *Blood* 90:3167-3172.

Salven, P., L. Teerenhovi, and H. Joensuu. 1999. A high pretreatment serum basic fibroblast growth factor concentration is an independent predictor of poor prognosis in non-Hodgkin's lymphoma. *Blood* 94:3334-3339.

Sanceau, J., and J. Wietzerbin. 2004. Downregulation of angiogenic factors in Ewing tumor xenografts by the combination of human interferon-alpha or interferon-beta with ifosfamide. *Annals of the New York Academy of Sciences* 1030:170-178.

Sawicki, G., A. Matsuzaki, and A. Janowska-Wieczorek. 1998. Expression of the active form of MMP-2 on the surface of leukemic cells accounts for their in vitro invasion. *Journal of cancer research and clinical oncology* 124:245-252.

Saydam, G., H.H. Aydin, F. Sahin, N. Selvi, G. Oktem, E. Terzioglu, F. Buyukkececi, and S.B. Omay. 2003. Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. *Leukemia research* 27:709-717.

Scavelli, C., G. Di Pietro, T. Cirulli, M. Coluccia, A. Boccarelli, T. Giannini, G. Mangialardi, R. Bertieri, A.M. Coluccia, D. Ribatti, F. Dammacco, and A. Vacca. 2007. Zoledronic acid affects over-angiogenic phenotype of endothelial cells in patients with multiple myeloma. *Molecular cancer therapeutics* 6:3256-3262.

Schmalfeldt, B., D. Prechtel, K. Harting, K. Spathe, S. Rutke, E. Konik, R. Fridman, U. Berger, M. Schmitt, W. Kuhn, and E. Lengyel. 2001. Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 7:2396-2404.

Sezer, O., C. Jakob, J. Eucker, K. Niemoller, F. Gatz, K. Wernecke, and K. Possinger. 2001a. Serum levels of the angiogenic cytokines basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in multiple myeloma. *European journal of haematology* 66:83-88.

Sezer, O., K. Niemoller, J. Eucker, C. Jakob, O. Kaufmann, I. Zavrski, M. Dietel, and K. Possinger. 2000. Bone marrow microvessel density is a prognostic factor for survival in patients with multiple myeloma. *Annals of hematology* 79:574-577.

Sezer, O., K. Niemoller, O. Kaufmann, J. Eucker, C. Jakob, I. Zavrski, and K. Possinger. 2001b. Decrease of bone marrow angiogenesis in myeloma patients achieving a remission after chemotherapy. *European journal of haematology* 66:238-244.

Sgonc, R., C. Fuerhapter, G. Boeck, R. Swerlick, P. Fritsch, and N. Sepp. 1998. Induction of apoptosis in human dermal microvascular endothelial cells and infantile hemangiomas by interferon-alpha. *International archives of allergy and immunology* 117:209-214.

Shalaby, F., J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman, and A.C. Schuh. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-66.

Shi, Q., S. Rafii, M.H. Wu, E.S. Wijelath, C. Yu, A. Ishida, Y. Fujita, S. Kothari, R. Mohle, L.R. Sauvage, M.A. Moore, R.F. Storb, and W.P. Hammond. 1998. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 92:362-367.

Sidky, Y.A., and E.C. Borden. 1987. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. *Cancer research* 47:5155-5161.

Singhal, S., J. Mehta, R. Desikan, D. Ayers, P. Roberson, P. Eddlemon, N. Munshi, E. Anaissie, C. Wilson, M. Dhodapkar, J. Zeddis, and B. Barlogie. 1999. Antitumor activity of thalidomide in refractory multiple myeloma. *The New England journal of medicine* 341:1565-1571.

Sipkins, D.A., X. Wei, J.W. Wu, J.M. Runnels, D. Cote, T.K. Means, A.D. Luster, D.T. Scadden, and C.P. Lin. 2005. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* 435:969-973.

Smith, S.M., D. Grinblatt, J.L. Johnson, D. Niedzwiecki, D. Rizzieri, N.L. Bartlett, and B.D. Cheson. 2008. Thalidomide has limited single-agent activity in relapsed or refractory indolent non-Hodgkin lymphomas: a phase II trial of the Cancer and Leukemia Group B. *British journal of haematology* 140:313-319.

Soker, S., S. Takashima, H.Q. Miao, G. Neufeld, and M. Klagsbrun. 1998. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92:735-745.

Soubrier, M., J.J. Dubost, A.F. Serre, J.M. Ristori, B. Sauvezie, P. Cathebras, J.C. Piette, A. Chapman, F.J. Authier, and R.K. Gherardi. 1997. Growth factors in POEMS syndrome: evidence for a marked increase in circulating vascular endothelial growth factor. *Arthritis and rheumatism* 40:786-787.

Steins, M.B., R. Bieker, T. Padro, T. Kessler, J. Kienast, W.E. Berdel, and R.M. Mesters. 2003. Thalidomide for the treatment of acute myeloid leukemia. *Leukemia & lymphoma* 44:1489-1493.

Stone, J., A. Itin, T. Alon, J. Pe'er, H. Gnessin, T. Chan-Ling, and E. Keshet. 1995. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15:4738-4747.

Streck, C.J., Y. Zhang, R. Miyamoto, J. Zhou, C.Y. Ng, A.C. Nathwani, and A.M. Davidoff. 2004. Restriction of neuroblastoma angiogenesis and growth by interferon-alpha/beta. *Surgery* 136:183-189.

Thyrell, L., S. Erickson, B. Zhivotovsky, K. Pokrovskaja, O. Sangfelt, J. Castro, S. Einhorn, and D. Grander. 2002. Mechanisms of Interferon-alpha induced apoptosis in malignant cells. *Oncogene* 21:1251-1262.

Tzankov, A., S. Heiss, S. Ebner, W. Sterlacci, G. Schaefer, F. Augustin, M. Fiegl, and S. Dirnhofer. 2007. Angiogenesis in nodal B cell lymphomas: a high throughput study. *Journal of clinical pathology* 60:476-482.

Vacca, A., D. Ribatti, L. Roncali, G. Ranieri, G. Serio, F. Silvestris, and F. Dammacco. 1994. Bone marrow angiogenesis and progression in multiple myeloma. *British journal of haematology* 87:503-508.

Veiga, J.P., L.F. Costa, S.E. Sallan, L.M. Nadler, and A.A. Cardoso. 2006. Leukemia-stimulated bone marrow endothelium promotes leukemia cell survival. *Experimental hematology* 34:610-621.

Vermeulen, P.B., L.Y. Dirix, M. Martin, J. Lemmens, and A.T. Van Oosterom. 1997. Serum basic fibroblast growth factor and vascular endothelial growth factor in metastatic renal cell carcinoma treated with interferon alfa-2b. *Journal of the National Cancer Institute* 89:1316-1317.

Vihinen, P., and V.M. Kahari. 2002. Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *International journal of cancer. Journal international du cancer* 99:157-166.

Westin, J., S. Rodjer, I. Turesson, A. Cortelezzi, M. Hjorth, and G. Zador. 1995. Interferon alfa-2b versus no maintenance therapy during the plateau phase in multiple myeloma: a randomized study. Cooperative Study Group. *British journal of haematology* 89:561-568.

Wu, W.Z., H.C. Sun, Y.F. Shen, J. Chen, L. Wang, Z.Y. Tang, G. Iliakis, and K.D. Liu. 2005. Interferon alpha 2a down-regulates VEGF expression through PI3 kinase and MAP kinase signaling pathways. *Journal of cancer research and clinical oncology* 131:169-178.

Yu, L., X. Wu, Z. Cheng, C.V. Lee, J. LeCouter, C. Campa, G. Fuh, H. Lowman, and N. Ferrara. 2008. Interaction between bevacizumab and murine VEGF-A: a reassessment. *Investigative ophthalmology & visual science* 49:522-527.

Zahiragic, L., C. Schliemann, R. Bieker, N.H. Thoennissen, K. Burow, C. Kramer, M. Zuhlsdorf, W.E. Berdel, and R.M. Mesters. 2007. Bevacizumab reduces VEGF expression in patients with relapsed and refractory acute myeloid leukemia without clinical antileukemic activity. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 21:1310-1312.